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(54) Title: ALLELE-SPECIFIC PRIMER EXTENSION ASSAY

(57) Abstract: The present invention provides methods of allele-specific primer extension useful for detecting mutations and genetic variations. In particular the invention provides a method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising: performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

ALLELE-SPECIFIC PRIMER EXTENSION ASSAY

The present invention relates to an improved assay for detecting mutations and genetic variations based upon the use of allele-specific primers.

Analysis of single nucleotide polymorphisms (SNPs) is useful in applications including mapping, linkage studies and pharmacogenomics. Consequently, a number of different techniques have been proposed to scan these sequence variations in a high-throughput fashion. Many of these methods originate from hybridization techniques to discriminate between allelic variants. High-throughput hybridization of allele-specific oligonucleotides can be performed on microarray chips (Wang et al. (1998) Science 280:1077), microarray gels (Yershov et al. (1996) Proc. Natl. Acad. Sci. USA 93:4913), or by using allele-specific probes (molecular beacons) in the polymerase chain reaction (PCR) (Tyagi et al. (1998) Nat. Biotechnol. 16:49). Other technologies which have been shown to be useful for SNP genotyping are minisequencing (Pastinen et al. (1997) Genome Res. 7:606), mass spectrometry (Laken et al. (1998) Nat. Biotechnol. 16:1352) and pyrosequencing (Ahmadian et al. (2000) Anal. Biochem. 280:103), the latter relying on incorporation of nucleotides by DNA polymerase with an enzymatic cascade converting the released pyrophosphate (PPi) into detectable light.

The use of pairs of allele-specific primers with alternative bases at the 3' end has been used to identify single base variations. Higgins et al. (1997) Biotechniques 23:710; Newton et al. (1989) Lancet 2:1481; Goergen et al. (1994) J. Med. Virol. 43:97; and Newton et al. (1989) Nucleic Acids Res. 17:2503. This method exploits the difference in primer extension efficiency by a DNA polymerase of a matched over a

mismatched primer 3'-end. Generally, a sample is divided into two extension reaction mixtures that contain the same reagents except for the primers, which differ at the 3'-end. The alternating primer is designed to match one allele perfectly but mismatch the other allele at the 3'-end. Because the polymerase differs in extension efficiency for matched versus mismatched 3'-ends, the allele-specific extension reaction thus provides information on the presence or absence of one allele.

The foregoing method of identifying single base variations using allele-specific primers with varying 3'-ends suffers from certain deficiencies. In particular, certain mismatches, such as G:T and C:A, are poorly discriminated by the DNA polymerase, leading to false positive signals (Day et al. (1999) Nucleic Acids Res. 27:1810). In these cases, DNA polymerase extends the mismatched primer-templates in the presence of nucleotides although, as the present inventors have shown, with slower reaction kinetics as compared to extension of the matched configuration. However, the kinetic difference is usually not distinguishable in end point analysis, such as allele-specific PCR.

The present invention solves the deficiencies of the prior art by providing a method of allele-specific extension that allows accurate discrimination between matched and mismatched configurations. The present methods are useful for high throughput SNP analysis.

The present invention provides methods of allele-specific primer extension useful for detecting mutations and genomic variations. In one embodiment, a nucleotide degrading enzyme, preferably apyrase, is included in the allele-specific primer reaction. In this method, nucleotides are degraded before extension in reactions having slow kinetics due to mismatches, but not in reactions having fast kinetics due to matches of primer and allelic target.

In another embodiment, the primers for the allele-specific primer extension reactions are designed such that the 3'-end base is complementary to the target, the penultimate (3'-1 end) base is allele-specific, and the base two positions from the 3'-end (3'-2 end) is non-complementary to (e.g. the same as) the target. When one mismatch is present (2 bases from the 3-end) the primer target duplex is stable and extension occurs. However, when two mismatches are present (at the 3'-1 and 3'-2 positions), duplex stability is disrupted and no detectable extension occurs.

Fig. 1 depicts the results of allele-specific extension for the three variants of the single nucleotide polymorphisms codon 72 (p53) and wiaf 1764. The SNP status, which was determined separately for each sample, and the extension primers are shown at the top of the Figure. Fig. 1a shows the raw data obtained using the luminometric assay without apyrase (top panel) and with apyrase (lower panel). The arrows point out the signal of pyrophosphate (0.02 :M) which was added to the reaction mixture prior to the nucleotide addition in all samples to serve as a positive control as well as for peak calibration. Fig. 1B shows the extension results of these samples using fluorescently labelled nucleotides spotted on a glass slide, with apyrase (lower panel) and without apyrase (top panel).

Fig. 2 is a schematic depiction of a method of apyrase mediated allele specific extension using barcodes as tags on the 5'-ends of allele-specific primers.

Fig. 3 is a schematic depiction of a method of allele-specific extension in solution followed by hybridization to a DNA microarray.

Fig. 4 is a schematic depiction of an apyrase mediated allele specific reaction in which Taq Man probes are used to measure primer extension.

Fig. 5 depicts raw data of an apyrase mediated

allele specific reaction utilizing a microarray format.

Fig. 6 depicts bioluminometric analysis of a SNP by double allele-specific primer extension on double stranded DNA.

Fig. 7 depicts allele specific extension using a primer having an introduced mismatch.

Fig. 8 is a schematic depiction of a method of detecting unknown mutations using partially overlapping extension primers.

Fig. 9 is a diagram showing the average intensities measured in an allele specific extension reaction.

The present invention relates to methods for detecting bases in a nucleic acid target. The present methods are useful for detecting mutations and genomic variations, and particularly single nucleotide polymorphisms (SNPs), and may be used with single stranded or double stranded DNA targets.

In one embodiment, the present invention provides a method of detecting a base at a predetermined position in a DNA molecule. The method utilizes DNA polymerase catalyzed primer extension. Pairs of primers are designed that are specific for (i.e. complementary to) an allele of interest, but that differ at the 3'-terminus, which position corresponds to the polymorphic nucleotide. In one primer, the 3'-terminus is complementary to the non-mutated nucleotide; in the other primer, the 3'-terminus is complementary to the mutated nucleotide. The primers are used in separate extension reactions of the same sample. Depending upon whether the mutation is present, the 3'-terminus of the primer will be a match or mismatch for the target. The DNA polymerase discriminates between a match and mismatch, and exhibits faster reaction kinetics when the 3'-terminus of the primer matches the template. Thus measurement of the difference in primer extension efficiency by the DNA polymerase of a matched over a mismatched 3'-terminus allows the determination of a

non-mutated versus mutated target sequence. In accordance with the present invention it has been found that the addition of a nucleotide degrading enzyme in the extension reaction minimizes the extension of mismatched primer configurations by removal of nucleotides before incorporation but allows extension when reaction kinetics are fast, i.e. in a matched configuration. The present invention thus reduces or eliminates false positive results seen in prior art methods.

In this aspect the present invention thus provides a method for detecting an allele-specific base at a predetermined position in a target nucleic acid molecule comprising providing a first and a second hybridization mixture, said first hybridization mixture comprising said target nucleic acid molecule, and a primer that hybridizes to a region of said target nucleic acid molecule and that has a 3'-terminus that is complementary to a non-mutated base at said predetermined position, said second hybridization mixture comprising said target nucleic acid molecule, and a primer that hybridizes to a region of said target nucleic acid molecule and that has a 3'-terminus that is complementary to a mutated base at said predetermined position; adding a primer extension reaction mixture to each of said first and second hybridization mixtures, said primer extension reaction mixture comprising a DNA polymerase, nucleotides, and a nucleotide degrading enzyme; and determining primer extension efficiency in each of said first and second mixtures, whereby greater efficiency in the mixture comprising the primer having a 3'-terminus that is complementary to the mutated base is indicative of the presence of the mutated base in the target nucleic acid, and whereby greater efficiency in the mixture comprising the primer having a 3'-terminus that is complementary to the non-mutated base is indicative of the presence of the non-mutated base in

the target nucleic acid.

In accordance with the present invention, samples may be prepared, primers synthesized, and primer-extension reactions conducted by methods known in the art, and disclosed for example by Higgins et al. (1997) Biotechniques 23:710; Newton et al. (1989) Lancet 2:1481; Goergen et al. (1994) J. Med. Virol. 43:97; and Newton et al. (1989) Nucleic Acids Res. 17:2503, the disclosures of which are incorporated by reference. SNP sites may be amplified prior to analysis, for example by PCR, including nested and multiplex PCR. SNP containing templates may be immobilized. This publication also describes how the nucleotide-degrading enzyme may be used.

The nucleotide degrading enzyme is preferably added after primer hybridization and preferably with the extension reaction mixture. For PCR reactions, a thermostable nucleotide degrading enzyme is used. As explained in detail in the Applicant's earlier application No. WO98/28440 relating to the use of nucleotide-degrading enzymes in pyrophosphate-detection based sequencing methods, any nucleotide-degrading enzyme may be used, capable of non-specifically degrading nucleotides. This publication also describes how the nucleotide-degrading enzyme may be used. Thus, the term "nucleotide-degrading enzyme" as used herein includes all enzymes capable of non-specifically degrading nucleotides, including at least nucleoside triphosphates (NTPs), but optionally also di- and mono-phosphates, and any mixture or combination of such enzymes, provided that a nucleoside triphosphatase or other NTP degrading activity is present. Although nucleotide-degrading enzymes having a phosphatase activity may conveniently be used according to the invention, any enzyme having any nucleotide or nucleoside degrading activity may be used, e.g. enzymes which cleave nucleotides at positions other than at the

phosphate group, for example at the base or sugar residues. Thus, a nucleoside triphosphate degrading enzyme activity is essential for the invention. Nucleoside di- and/or mono-phosphate degrading enzymes or enzyme activities are optional and may be used in combination with a nucleoside tri-phosphate degrading enzyme activity. Suitable such enzymes include most notably apyrase which is both a nucleoside diphosphatase and triphosphatase, catalysing the reactions NTP → NMP + 2Pi and NTP → NDP + Pi (where NTP is a nucleoside triphosphate, NDP is a nucleoside diphospate, NMP is a nucleotide monophosphate and Pi is phosphate). Apyrase may be obtained from Sigma Chemical Company. Other suitable nucleotide triphosphate degrading enzymes include Pig Pancreas nucleoside triphosphate diphosphohydrolase (Le Bel et al., 1980, J. Biol. Chem., 255, 1227-1233). Further enzymes are described in the literature.

Different combinations of nucleoside tri-, di- or monophosphatases may be used. Such enzymes are described in the literature and different enzymes may have different characteristics for deoxynucleotide degradation, eg. different Km, different efficiencies for a different nucleotides etc. Thus, different combinations of nucleotide degrading enzymes may be used, to increase the efficiency of the nucleotide degradation step in any given system. For example, in some cases, there may be a problem with contamination with kinases which may convert any nucleoside diphosphates remaining to nucleoside triphosphates, when a further nucleoside triphosphate is added. In such a case, it may be advantageous to include a nucleoside disphosphatase to degrade the nucleoside diphosphates. Advantageously all nucleotides may be degraded to nucleosides by the combined action of nucleoside tri-, di- and monophosphatases.

Generally speaking, the nucleotide-degrading enzyme

is selected to have kinetic characteristics relative to the polymerase such that nucleotides are first efficiently incorporated by the polymerase, and then any non-incorporated nucleotides are degraded. Thus, for example, if desired the K_m of the nucleotide-degrading enzyme may be higher than that of the polymerase such that nucleotides which are not incorporated by the polymerase are degraded. Thus, the nucleotide-degrading enzyme or enzymes are simply included in the polymerase reaction mix. The amount of nucleotide-degrading enzyme to be used, may readily be determined for each particular system, depending on the reactants selected, reaction conditions etc.

As mentioned above, the nucleotide-degrading enzyme(s) may be included during the polymerase reaction step. This may be achieved simply by adding the enzyme(s) to the polymerase reaction mixture prior to or simultaneously with the polymerase reaction (i.e. the chain extension or nucleotide incorporation), e.g. prior to or simultaneously with, the addition of the polymerase and/or nucleotides to the sample/primer.

In one embodiment, the nucleotide-degrading enzyme(s) may simply be included in solution in a reaction mix for the polymerase reaction, which may be initiated by addition of the polymerase or nucleotide(s).

In a preferred embodiment of the present method, the nucleotide degrading enzyme is apyrase, which is commercially available, for example from Sigma Chemical Co., St. Louis, Mo. USA. Those of ordinary skill in the art can determine a suitable amount of apyrase or other nucleotide degrading enzyme to degrade unincorporated nucleotides in extension reactions having slow kinetics due to a mismatch at the 3'-terminus of the primer. For example, a typical 50 :1 extension reaction mixture may contain for 1 to 15 mU, and preferably from 5 to 10 mU, of apyrase.

In the present invention, primer extension can be measured, and thus the ratio of extension using the primer having a matched 3'-terminus and extension using the primer having an unmatched 3'-terminus determined, by methods known in the art. If two primers are used in each reaction, PCR products can be generated and measured. Other methods of measuring primer extension include mass spectroscopy (Higgins et al. (1997) Biotechniques 23:710), luminometric assays, in which incorporation of nucleotides is monitored in real-time using an enzymatic cascade, (Nyren et al. (1997) Anal. Biochem. 244:367), fluorescent assays using dye-labelled nucleotides, or pyrosequencing as described by Ronaghi et al. (1998) Science 281:363, and Ahmadian et al. (2000) Anal. Biochem. 280:103.

It has been found that the method of the invention may advantageously be performed using labelled nucleotides in the primer extension reactions to provide a convenient way of monitoring (e.g. detecting or measuring) the primer extension reactions; it may be detected whether or not incorporation has taken place by detecting the label carried on the nucleotide, or the relative or respective rates (or efficiencies) of nucleotide incorporation.

In a preferred aspect, the invention thus provides a method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising:

performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

As explained above, this method of the invention

may be used to detect a base change at a particular pre-determined position, by determining which base is present at that position. The method may thus be used to detect mutations, or genetic variation (e.g. allelic variation) based on base changes. The base detected may thus be allele-specific. The method has particular utility in the detection of single base changes or single nucleotide polymorphisms (SNPs). The primers used may thus be allele-specific. The method may be used to detect base changes (e.g. polymorphisms or mutations) which are known or unknown. Thus known or unknown base changes may be identified.

As discussed above, allele-specific assays based on the use of allele-specific primers are well known in the art and widely described in the literature. Any such "allele-specific" primers may be used as the "base-specific" primer in the method of the invention. Thus, the base-specific primer is designed to be specific for, or selective for, a particular base, for example a base the presence or absence of which it is desired to detect. Such a primer will only be extended (e.g. detectably extended) or will have a greater extension efficiency (or higher rate of extension) in the presence of that base in the target (i.e. pre-determined) position in the target nucleic acid molecule which acts as template in the primer extension reaction, and will not be extended (or will be extended at a lower rate or with reduced efficiency) in the absence of that base. Such primers therefore "match" or "mismatch" the particular base for which they are selective or specific.

Most conveniently, as discussed above, such base-specific, or allele-specific primers may be designed to comprise a "match" or "mismatch" for the target base at their 3' end (i.e. at the 3' terminus). Thus 3' base (i.e. the base at the 3' terminus) of the primer is designed either to match (i.e. to be complementary) to a

particular base (e.g. a particular allele, or mutation) or mismatch it (i.e. be non-complementary). As explained above, in the context of a mutation, in one primer the 3' base may be complementary to the non-mutated (e.g. wild-type) base and in a second primer i.e. may be complementary to a mutated base (e.g. the mutation it is desired to detect).

Thus, in the context of a known mutation or biallelic variation (e.g. SNP), for example, two primers may be used (so called allele-specific primers) the first "matched" to the one base which may occur, and the second "matched" to the other base which may occur. Where more than one base may occur, more than two base-specific primers may be used, each specific for (or "matched to") each of the bases which may occur.

The method of the invention thus comprises the use of at least two (or two or more) base-specific primers, e.g. 2, 3 or 4 primers. As discussed further below, the method of the invention may be adapted to the detection of unknown mutations, simply by providing primers specific for each of the 4 bases (A, T, C or G) which may occur at a particular, (i.e. pre-determined) position. Thus, each position in a target sequence may be "scanned" for mutations.

Although, as mentioned above, the use of base-specific primers which differ at their 3' terminus represents one way of performing the method, the method of the invention is not so limited, and alternative primer designs are also possible, as again is well known and widely described in the art. Thus, allele-specific (or base specific) primers according to the present invention may comprise a match (or mismatch) for the target base at other positions, for example at the 3'-1 position of the primer (i.e. the penultimate base of the primer). Thus the "allele-specific" (or "base-specific") base in the primer need not be the 3' base, but it may be present at other positions e.g. the -1 or

-2 (ante-penultimate) position. In addition, the primer may also comprise other modifications (e.g. other mismatches) at other positions to aid or improve "base-specify" (i.e. discrimination between the "matched" and "mismatched" situation). Again, such principles of primer design are also discussed in the literature. A further aspect of the present invention is based on modified primer design and is discussed further below.

The primer extension reactions may be performed as described above. For example the target nucleic acid molecule may be contacted with each said base-specific primer in conditions under which hybridisation (or annealing) of the primers, and subsequently primer extension, may occur. Conveniently, "hybridization mixtures" comprising template (i.e. target nucleic molecule) and primer may be prepared (i.e. 1 to 4 hybridisation mixtures depending on the number of primers used). Polymerase and nucleotides for the performance of the primer extension reaction may then be added. As explained above, the nucleotide degrading enzyme may be included in the primer extension reaction mixture, or added separately.

The primer extension reactions performed using the different base-specific primers are then compared. More particularly, in this comparison step, the rates of extension may be compared (e.g. the respective or relative rates). Thus, efficiencies of the primer extension reactions may be compared.

The respective rates, or efficiencies, of the primer extension reactions may be determined for each of the different primers and compared. A greater efficiency, or higher extension rate, is indicative of the presence of the base for which the primer is specific (i.e. "matched"). Thus, if a detection (i.e. base-specific) primer matches the template (i.e. the target base) a high extension rate (or efficiency) will be observed. If the primer is not a matched primer, the

extension rate (or efficiency) will be lower. The difference in primer extension rate (or efficiency) over a matched or mismatched primer can be used for discrimination between bases, and thus to determine which base is present at said pre-determined position.

It has been found that the use of labelled nucleotides, represent a particularly advantageous way of detecting, and comparing, the primer extension reactions. Thus, the nucleotides used for incorporation in the primer extension reaction may carry a label, preferably a detectable label, which may be used to detect whether or not the nucleotide has been incorporated, or to follow or monitor the primer extension reaction to detect the rate or efficiency of nucleotide incorporation. Such a label may be any label giving or leading to a detectable signal (which may be directly or indirectly detectable, for example a moiety which takes part in a reaction (e.g. an enzymic or antibody-based reaction) which generates a detectable signal). Directly detectable labels are preferred, and especially labels based on dyes which may be spectrophotometrically or fluorescently detectable. Fluorescent labels are especially preferred.

Labelled nucleotides for use in sequencing are well known in the art and widely described in the literature and any of these may be used. WO 00/53812, for example, describes, *inter alia*, methods of sequencing based on dye-labelled (particularly fluorescently labelled) nucleotides, which may be used according to the present invention. See also US-A-6,087,095.

In preferred embodiments, the label, for example the dye (e.g. fluorophore), may be attached to the nucleotide via a cleavable linker which can be cleaved to remove the label from the nucleotide when desired. Cleavage may be accomplished, for example, by chemical reaction (e.g. acid or base treatment) or by oxidation or reduction of the linkage or by light treatment, or

enzymically, depending on the nature of the linkage. Such cleavable linkages are described in detail in WO 00/53812. A preferred example is a disulphide linkage which may be cleaved by reduction, e.g. with a reducing agent such as dithiothreitol. Dyes, for example fluorophores, e.g. cyanine fluorophores (e.g. cyanine 3 or cyanine 5 fluorophores) may be conjugated via disulphide bonds to a nucleotide as described in WO 00/53812. Advantageously the label may be attached to the 3' moiety of the deoxyribose group of a nucleotide, such that cleavage and removal of the label group from the nucleotide generates a 3' OH group. Modified labelled nucleotides known as "false terminators" where a blocking group may be removed from the modified nucleotide are also described in EP-A-0745688 and US-A-5,302,509.

The present method may be performed in a solid phase microarray format, for example on a chip, whereby samples are extended in solution followed by hybridization to a microarray. (Fig. 3). Alternatively, the method may be performed in a microarray format in which the primers or samples (templates) are immobilised. Allele-specific primer extension on microarrays is known in the art and described for example by Pastinen et al. (2000) Genome Research 10:1031. The method may also be performed a liquid phase assay using barcodes as tags on the 5'-ends of allele-specific primers as described by Fan et al. (2000) Genome Res. 10:853. By using barcodes, a liquid-phase multiplex apyrase mediated allele specific extension of a set of SNPs may be performed in a single tube (if the barcodes on the matched and mismatched primers are different) or in two tubes (if the barcodes on the matched and mismatched primers are identical). After primer extension, the extension products can be hybridized to barcodes on the chip. A modular probe as described by O'Meara et al. (1998) Anal. Biochem.

255:195 and O'Meara et al. (1998) J. Clin. Microbiol. 36:2454 may be utilized to improve the hybridization efficiency. The modular probe hybridizes to its complementary segment on the immobilized oligonucleotide and improves the hybridization of a barcode that is immediately downstream.

The present method may also be performed using "Taq Man" probes, which are probes labelled with a donor-acceptor dye pair that functions via fluorescence resonance transfer energy (FRET) as described by Livak et al. (1995) PCR Methods Appl. 4:357. When Taq Man probes are hybridized to a target, the fluorescence of the 5'-donor fluorophore is quenched by the 3'-acceptor. When the hybridized probe is degraded, the 5'-donor dye dissociates from the 3'-quencher, leading to an increase in donor fluorescence.

After PCR amplification, a Taq Man probe is used which hybridizes 15 to 20 bases downstream of a 3'-end allele-specific primer. Apyrase-mediated allele specific extension is then performed. In the case of a matched primer - template, DNA polymerase extends the primer and degrades the Taq Man probe by its 5'-nuclease activity, thus leading to increased donor fluorescence. In the mismatched case, apyrase degrades the nucleotides and the fluorescence level remains unchanged. Such an assay is depicted in Figure 4.

Extension products may also be distinguished by mass difference or by the use of double-stranded-specific intercalating dyes.

In another embodiment of the present invention, allele specific primer extension with a nucleotide degrading enzyme is performed using a double stranded DNA template. If double stranded DNA is generated by PCR, the excess of primers, nucleotides and other reagents must first be removed by methods known in the art such as treatment with alkaline phosphatase and exonuclease I. Two pairs of allele-specific primers are

used. One pair is complementary to the forward strand and one to the reverse strand. The primer pairs differ in their 3'-ends as described above. Allele-specific extension is performed on both strands of the double-stranded DNA.

As mentioned above, in one embodiment, the present invention provides a method of detecting a mutation in a DNA molecule. In this method, the mutation is not pre-defined, as in a SNP (i.e. it is unknown). The method utilizes DNA polymerase catalyzed primer extension. Four primers are designed that each comprise a region that is specific for (i.e., complementary to) a target nucleic acid of interest or to a region interest in a target nucleic acid, e.g. a particular stretch of nucleotides, but that differ (e.g. at the 3'-terminus) such that primer has a different nucleotide base (A, C, G or T) at a "base specific" (or "allele-specific" position), for example at the 3'-terminus. As mentioned above, alternatively the "allele-specific" base or different nucleotide may be present at another position, e.g. the 3'-1 position. The primers are used in separate extension reactions of the same sample of target nucleic acid. Depending upon which base is present in the target nucleic acid at the position corresponding to the 3'-terminus of the primer (or any other "allele-specific" position), only one of the primers will match the target. The DNA polymerase discriminates between a match and mismatch, and exhibits faster reaction kinetics when the 3'-terminus allele-specific base of the primer matches the template. Thus measurement of the difference in primer extension efficiency by the DNA polymerase of the matched over the three mismatched 3'-termini allows determination of the base at the position in the target nucleic acid that corresponds to the 3'-terminus of the primer (or analogously, at any other "base or allele-specific" position). Accordingly, if a previously unknown mutation is present in the target

nucleic acid, the mutation is detected by virtue of one of the four primers "matching" the mutated target, and that primer exhibiting the greatest extension efficiency (or highest extension rate). Thus, the identity of the target base may be determined by determining which primer is extended (i.e. which base is incorporated) with the greatest rate or efficiency (or highest extension rate). In accordance with the present invention it has been found that the addition of a nucleotide degrading enzyme in the extension reaction minimizes the extension of mismatched primer configurations by removal of nucleotides before incorporation, but allows extension when reaction kinetics are fast, i.e., in a matched configuration, thereby reducing or eliminating false positive results.

Samples may be prepared, primers synthesized, and primer-extension reactions conducted by methods known in the art as described hereinabove. Primers may be immobilized. Samples of target nucleic acids may be amplified prior to analysis, for example by PCR, including nested and multiplex PCR. Target nucleic acids may be immobilized.

In a preferred embodiment of such a mutation-detection method, each position in a target nucleic acid (or in a selected or target region) may be evaluated by using overlapping sets of extension primers. Thus, each position in a target region may be scanned for mutations using this method of the invention, simply by appropriately selecting or designing a detection primer such that the base at the selected "allele-specific" position in the primer (e.g. the 3'-terminus) corresponds to, or is complementary to the base to be scanned, i.e. the target base at the desired position. The primers may be spotted onto a surface to provide an array, followed by hybridization to target nucleic acid, extension, measurement of extension, and comparison of extensions.

In another embodiment of the present invention, allele specific extension is performed using a pair of primers in which the 3'-end base is complementary to the target, the penultimate (3'-1 end) base is allele-specific (mutated or nonmutated) and the base two positions from the 3'-end (3'-2 end) is non-complementary to (for example the same as) the target. When the allele-specific base in the primer matches the target template, i.e. one mismatch is present (2 bases from the 3'-end), the primer-target duplex is stable and extension occurs. When the allele-specific base does not match the target, i.e. two mismatches are present (at the 3'-1 and 3'-2 positions), duplex stability is disrupted and no detectable extension occurs. The assay may be performed in all the embodiments as described above, in the presence or absence of a nucleotide degrading enzyme. An example of the method is depicted in Fig. 7.

All references cited herein are incorporated herein in their entirety.

The following non-limiting examples serve to further illustrate the present invention.

Example 1

Apyrase Mediated Allele Specific Extension

Experimental protocol

Samples, PCR and single strand preparation

Human genomic DNA was extracted from twenty-four unrelated individuals. Two duplex PCRs were performed to amplify four SNPs. The SNPs were wiaf1764 (A/C) on chromosome 9q, codon 72 (C/G) on the p53 gene (Ahmadian et al. (2000) Anal. Biochem. 280:103) nucleotide position 677 (C/T) on the MTHFR gene (Goyette et al. (1998) Mamm. Genome 9:652, erratum in (1999) Mamm. Genome 10:204) and nucleotide position 196 (A/G) on the GPIIIa gene (Newman et al. (1989) J. Clin. Invst.

83:1778). The outer duplex PCR for wiaf 1764 and p53 gene (94°C 1 min, 50°C 40s and 72°C 2 min for 35 cycles) was followed by specific inner PCRs (94°C 1 min, 50°C 40s and 72°C 1 min for 35 cycles) generating ~80 bp fragments for each SNP (Table 1). The outer duplex PCR condition for MTHFR and GPIIIa genes was 95°C 30s, 60°C 1 min and 72°C 1 min. This was followed by individual inner PCRs (95°C 1 min, 66°C 50 sec and 72°C 2 min) (Table 1). The outer and inner amplification mixtures comprised of 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.1% (v/v) Tween 20, 0.2 mM dNTPs, 0.1 μM of each primer and 1 unit of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Connecticut, USA) in a total volume of 50 μl. Five microliters of total human DNA (1 ng/μl) were used as outer PCR template. In the inner PCR, one of the primers in the respective set was biotinylated at the 5'-end to allow immobilization. 40 μl biotinylated inner PCR products were immobilized onto streptavidin-coated super paramagnetic beads (Dynabeads M280; Dynal, Oslo, Norway). Single-stranded DNA was obtained by incubating the immobilized PCR product in 12 μl 0.1 M NaOH for 5 min. The immobilized strand was then used for hybridization to extension primers (Table1).

Apyrase mediated allele-specific extension using a bioluminometric assay

The immobilized strand was resuspended in 32 μl H_2O and 4 μl annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate). The single stranded DNA was divided into two parallel reactions in a microtiter-plate (18 μl/well) and 0.1 μM (final concentration) of primers were added to the single stranded templates in a total volume of 20 μl. Allele discrimination between the allelic variants was investigated by the use of Klenow DNA polymerase using two separate SNP primers that only differed in the 3'-end position (Table 1). Hybridization was performed by incubation at 72°C for 5

min and then cooling to room temperature. The content of each well was then further divided into two separate reactions for comparison of extension analysis with and without apyrase. Extension and real-time luminometric monitoring was performed at 25°C in a Luc96 pyrosequencer instrument (Pyrosequencing, Uppsala, Sweden). An extension reaction mixture was added to the single stranded DNA (10 µl) with annealed primer (the substrate) to a final volume of 50 µl. The extension reaction mixture contained 10 U exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden), 4 µg purified luciferase/ml (BioThema, Dalarö, Sweden), 15 mU recombinant ATP sulfurylase, 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine serum albumin (BioThema), 1 mM dithiothreitol, 10 µM adenosine 5'-phosphosulfate (APS), 0.4 mg polyvinylpyrrolidone /ml (360 000), 100 µg D-luciferin/ml (BioThema) and 8 mU of apyrase (Sigma Chemical Co., St. Louis, MO, USA) when applicable. Prior to nucleotide addition and measuring of emitted light, pyrophosphate (PPi) was added to the reaction mixture (0.02 µM). The PPi served as a positive control for the reaction mixture as well as peak calibration. All the four nucleotides (Amersham Pharmacia Biotech, Uppsala, Sweden) were mixed and were dispensed to the extension mixture (1.4 µM, final concentration). The emitted light was detected in real-time.

Apyrase mediated allele-specific extension using fluorescent labeled nucleotide

The single stranded templates were prepared using magnetic beads as outlined above. The immobilized strand was resuspended in 100 µl H_2O and 12 µl annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate). The single stranded DNA was divided into two parallel reactions in a microtiter-plate (56 µl/well) and 0.1 µM

(final concentration) of primers (Table 1) were added to the single stranded templates in a total volume of 60 μl. Hybridization was performed by incubation at 72°C for 5 min and then cooling to room temperature. The reaction mixture was then further divided into two separate reactions (30 μl) for direct comparison of the extensions with and without apyrase. The extension mixture contained 20 U exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Pharmacia Biotech), Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine serum albumin (BioThema),1 mM dithiothreitol and optionally 8 mU of apyrase (Sigma Chemical Co.) in a total volume of 70 μl. The extension mixture also contained nucleotides, dATP, dGTP, dTTP and dCTP (1.4 μM final concentration), where dCTP was labeled with Cy5 or Cy3 (Amersham Pharmacia Biotech). The reaction was carried out for 15 min at room temperature. The resulting extension products were washed twice with Tris-EDTA and the non-biotinylated extension products were isolated by adding 25 μl 0.1 M NaOH. The eluted strand was neutralized by 12.5 μl 0.2 M HCl and was printed on a glass slide by the GMS 417 Arrayer (Genetic MicroSystem, USA) and then was scanned using GMS 418 Scanner (Genetic MicroSystem, USA). The obtained results were analyzed using GenePix2.0 software (Axon Instruments, USA).

Apyrase mediated allele-specific extension using fluorescent labeled nucleotide evaluated in a microarray format

After amplification, the amplicons of wiaf 1764 were immobilized onto streptavidin coated beads as outlined above. The biotinylated inner primer was primer 3 and not primer 4 (Table 1) with the reason that for this assay the eluted strand is the extension substrate, thus to have the same match and mismatch configurations, the biotinylated inner primer was

changed. The immobilized PCR product of wiaf 1764 was incubated in 20 µl 0.1 M NaOH for 5 min. The eluted strand was neutralized by 10 µl 0.2 M HCl and 4 µl annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate) was added. The eluted and neutralized single stranded DNA was divided into two parallel reactions in a microtiter-plate (17 µl/well) and extension primers (0.1 µM) were added. Hybridization was performed by incubation at 72°C for 5 min and then cooling to room temperature. Here, the extension primers were modified to have amino groups at the 5'-end to allow covalent binding to pre-activated Silyated Slides (Cel Associates Inc, Texas, USA). In order to improve hybridization and extension the primers were extended with a 15-mer spacer (T_{15}) in the 5'-end (Table 1). One microliter of the primer-template hybrid was manually spotted on Silyated Slides (Cel Associates Inc) and the covalent coupling was performed in a humid chamber at 37°C for 16h. After coupling, 1 mU apyrase (1 µl) was added to each spot. A polymerization mixture was prepared and 4 µl was added to the spots immediately after addition of apyrase. The polymerization mixture contained Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine serum albumin (BioThema), 1 mM dithiothreitol, 0.08 µM (final concentration) dCTP labeled with Cy3 (Amersham Pharmacia Biotech) and 1 U exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Pharmacia Biotech). Polymerization was allowed to proceed for 15 min and the microarray slide was washed briefly with water and then scanned using GMS 418 Scanner (Genetic MicroSystem, USA). The data was analyzed by using GenePix2.0 software (Axon Instruments, USA).

Pyrosequencing

Single stranded DNA with annealed sequence primer (the substrate) (Table 1) were used for pyrosequencing. Real-time pyrosequencing was performed at 28°C in a

total volume of 50 μl in an automated 96-well PyroSequencer using PSQ™ SNP 96 enzymes and substrates (Pyrosequencing AB, Uppsala, Sweden).

Results

Two different approaches of apyrase mediated allele-specific extensions were investigated. The assays included a luminometric assay and two assays based on fluorescent labeled nucleotides. All the results obtained with these assays were confirmed by pyrosequencing. Four SNPs with the eight alternative 3'-end primer-template configurations were investigated (Table 1 and Table 2). The SNPs were codon 72 of the p53 gene (C or G), wiaf 1764 (G or T), nucleotide position 677 in the MTHFR gene (C or T) and nucleotide position 196 in the GPIIIa gene (G or A). Thus, using two alternative allele-specific primers for each SNP, the following mismatches were possible; G-G and C-C for codon 72 (p53 gene), A-G and C-T for wiaf 1764, A-C and G-T for polymorphic position on the MTHFR gene, T-G and C-A for the SNP on the GPIIIa gene (Table 2).

Figure 1A shows the results of the luminometric assay with and without apyrase, for codon 72 of the p53 gene and wiaf 1764. When codon 72 is homozygous G (sample g1) (see Table 2), the mismatch signal is as high as the match signal but the slope of the curve indicates slower reaction kinetics (Figure 1A top panel, extension 2). The same was observed for wiaf 1764 homozygous T (sample g055) (see Table 2). However, when apyrase was included in the allele-specific extension of these samples (Figure 1A, lower panel) a dramatic difference was observed. The previous high signals for mismatch configurations, disappeared with the addition of apyrase. The extension ratios were calculated by taking the ratio of the high versus the low end-point signals. For example, the ratio for sample g1 in the p53 gene was 1.1 without apyrase and 13.9 with apyrase

and for the sample g055 in wiaf 1764 the ratio was 1 without apyrase and 5 with apyrase (Table 2). This clearly shows that addition of apyrase affects the extension and simplifies interpretation. In these assays an extension ratio below or equal to 2 was interpreted as a sample being heterozygous. If the ratio was above or equal to 2.5 the SNP was scored as homozygous with the nucleotide at the 3'-end of the primer that produced the higher signal. Ratios between 2 and 2.5 were interpreted as uncertain. As shown in Figure 1A and Table 2, in all cases with addition of apyrase the extension signals and extension ratios resulted in a correct genotype with the luminometric assay as compared to the pyrosequencing data. In fact, the extension ratios for all heterozygous SNPs were between 1.1 and 1.4 and the lowest extension ratio for a homozygous sample was 5. In contrast, without the inclusion of apyrase, five out of eight mismatches contributed with so high extension signals that the SNPs were wrongly scored (ratios in bold). The primer-template mismatches that were extended in these cases were G-G, C-T, G-T, T-G and C-A. Figure 1B also shows the raw data of similar extensions on DNA immobilized on magnetic beads using fluorescent detection using a labeled nucleotide instead of a luminometric detection system. Thus, extension is performed by the same polymerase and the same primers, meaning that the discrimination behavior should be as in the luminometric assay. The results (extension ratios) are in good agreement with the luminometric assay (Table 2). Without apyrase the same mismatches gave high fluorescent signals leading to that the same homozygous samples were wrongly scored as heterozygous (ratios in bold in Table 2). However in presence of apyrase, no ambiguities or discordant results were observed (Figure 1B lower panel and Table 2).

The foregoing example demonstrates that apyrase

aids in the discrimination of mismatches in allele-specific extension. The extensions in these cases were performed on magnetic beads. The use of this technology in a microarray format was also evaluated. The SNP wiaf 1764 were amplified in 3 samples (g011, 119 and g055) and single stranded templates were obtained by immobilization of the PCR product onto magnetic beads. The eluted non-biotinylated strand was used in the subsequent extension experiments. Extension primers were modified to have a free amino group in the 5'-end as well as a 15 nucleotide long oligo dT spacer. The extension primers and the single strand target were hybridized, printed and then covalently coupled to the activated glass slides. The extension reaction mixtures were then added to microarray. Figure 5 shows the raw-data of the analysis without and with apyrase. A direct comparison of the raw data obtained for samples of wiaf 1764 using both assays shows that the results of extension on chip are correct when apyrase is used while the same homozygous sample as in the other two assays (g055) has led to a high mismatch signal when apyrase was not used.

As shown above, a major advantage of using apyrase mediated allele specific extension (AMASE) is that the technique is applicable for high throughput genotyping. The present method may also be performed using barcodes (Fan et al. (2000) Genome Res. 10:853) as tags on the 5'-ends of allele-specific primers. In this way a multiplex AMASE of a set of SNPs is performed in a single tube (if the barcodes on the match and mismatch are different) or in two tubes (if the barcodes on the match and mismatch are identical). After performance of AMASE, the double-stranded AMASE products are heat separated and hybridized to barcode complementary oligonucleotides on the chip (Figure 2). To improve the hybridization efficiency, at the hybridization step a modular probe is utilized (O'Meara et al. (1998) J.

Clin. Microbiol. 36:2454). The modular probe can hybridize to its complementary segment of the immobilized oligonucleotide and improve the hybridization of barcode that is immediately downstream.

In conclusion, this example shows that single nucleotide polymorphisms can rapidly be scored with allele-specific primers in extension reactions. Furthermore, the present method can be used in a high-throughput format using microarrays.

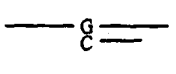
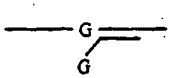
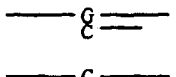
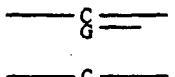
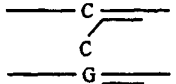
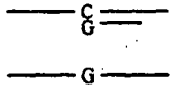
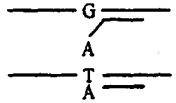
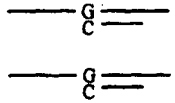
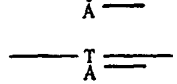
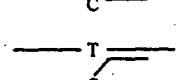
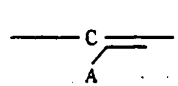
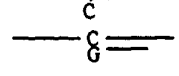
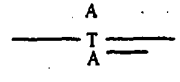
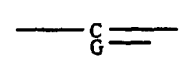
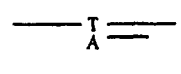
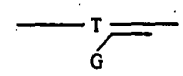
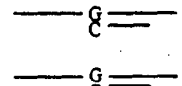
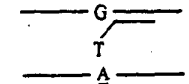
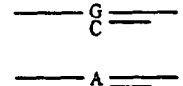
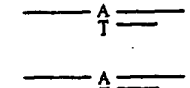
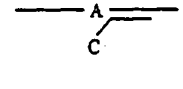
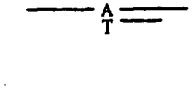
Table 1. List of the primers.

SNP	Primer	5' 3'
wiaf1764	1	AGTGAAAACATTGAAAACACA
	2	AATGTTTTCACTGTCATAAAG
	3	TCCAATGTGTGAAAAATATATAC
	4-Biotin	AGAACACATACGTTTTACCA
	Extension1	ACTCCCTTCAGATCA
	Extension2	ACTCCCTTCAGATCC
	Amino-Extension1	TTTTTTTTTTTTTTTATACAAC ACTCCCTTCAGATCA
	Amino-Extension2	TTTTTTTTTTTTTTTATACAAC ACTCCCTTCAGATCC
	Seq	CATTTGTTAAGCTTTT
p53 codon 72	1	ATGCTGTCCCCGGACGA
	2	CAGGAGGGGGCTGGTG
	3-Biotin	TCCAGATGAAGCTCCCAG
	4	AGGGGCCGCCGGTGTA
	Extension1	GCTGCTGGTGCAGGGGCCACGC
	Extension2	GCTGCTGCTGCAGGGGCCACGG
	Seq	GCTGCTGGTGCAGGGGCCA
mthfr	1	CCTGACTGTCATCCCTATTGGCAG
	2	GGGACGATGGGGCAAGTGATG
	3-Biotin	GCTGACCTGAAGCACTTGAAGGAG
	4	GCCTCAAAGAAAAGCTGCGTG
	Extension1	GCTGCGTGATGATGAAATCGA
	Extension2	GCTGCGTGATGATGAAATCGG
	Seq	AAGCTGCGTGATGATGAAA
gp3a	1	GCCATAGCTCTGATTGCTGGACTTC
	2	GCCTCACTCACTGGGAACTCGATG
	3	GCTGGACTTCTCTTTGGGCTCCTG
	4-Biotin	ACAGTTATCCTTCAGCAGATTCTCCTT
	Extension1	TCTTACAGGCCCTGCCTCC
	Extension2	TCTTACAGGCCCTGCCTCT
	Seq	CCTGTCTTACAGGCCCTGCC

1 and 2 = primers used in the outer PCR. 3 and 4 = primers used in the inner PCR. 1 and 3 are upstream primers and 2 and 4 are downstream primers. Biotin indicates the biotinylated primer in each set. Extension1 and Extension2 refer to the primers used as

match and/or mismatch with the alternating base in the 3'-end. Amino-Extension refer to the primers used on microarray. Seq = primers used for pyrosequencing. Notice that when the Amino-Extension primers (for wiaf 1764) are used, the biotinylated inner primer was primer 3 and not primer 4

Table 2. Summary of extension results

SNP	Sample	Sequencing Result	Schematic Representation of Configurations		Allele-Specific Extension Ratio (high /low)			
					Bioluminescence		Fluorescent	
			Extension 1	Extension 2	- apyrase	+ apyrase	- apyrase	+ apyrase
p53 (G/C)	g1	G/G			**1.1**	13.9	**1.2**	4.9
	116	G/C			1.2	1.1	1.4	1.4
	123	C/C			**2.6**	12	**2.3**	10.5
wiaf 1764 (G/T)	g011	G/G			8.3	8	15.5	3.9
	119	T/G			1.2	1.1	1.3	1.5
	g055	T/T			**1**	5	**1.1**	8.2
MTHFR (C/T)	1001	C/C			3.5	12	20	35
	1055	T/C			1	1.3	1.1	1.3
	1004	T/T			**1.1**	12	**2.3**	13
GPIIIa (G/A)	1267	G/G			**1.1**	6.5	**1.1**	3.5
	1001	G/A			1.2	1.4	1.4	1.1
	1055	A/A			**1.4**	10	**1.1**	7.1

Example 2

Apyrase mediated allele-specific extension on DNA microarrays

Apyrase mediated allele-specific extension (AMASE) for genotyping on DNA microarrays is described in this report. The method involves extension of the DNA samples in solution followed by hybridization to the DNA microarray as illustrated in Figure 3.

Materials and Method

Microarray preparation

Amino linked oligonucleotide capture probes suspended at a concentration of 20μM in 3X SSC/0.01% sakrosyl were spotted using a GMS 418 arrayer (Affymetrix, USA) on silylated slides (CEL Associates, Houston, TX). Printed arrays were allowed to dry for 12 hours at room temperature followed by post processing to reduce unreacted aldehyde groups thereby minimising non-specific binding of target. Briefly the slides were washed twice in 0.2% SDS for 2 minutes, twice in dH_2O for 2 minutes and treated with sodium borohydride (0.75g $NaBH_4$ dissolved in 225 ml PBS and 75 ml 100% ethanol) for 5 minutes. The arrays were then washed in 0.2% SDS three times for 1 minute, rinsed in H2O and dried by centrifugation for 1 minute at 500g. Prior to use in hybridization, the arrays were prehybridized with buffer containing 5X Denharts solution, 6X SSC, 0.5% SDS and 0.1 μg/μl herring sperm DNA at 50 °C for 15 minutes followed by a brief rinse in dH_2O.

Oligonucleotides

The capture probes were synthesised with an amino group at the 5' end to facilitate covalent immobilisation on the glass slide. A carbon spacer was also synthesised at the 3' end to prevent any possible extension of the capture probe during hybridization,

albeit unlikely due to the high salt conditions. The sequences of the capture probes and the allele specific extension primers are listed in Table 3.

DNA preparation

PCR was carried out on human genomic DNA as previously described (Ahmadian *et al.* (2000) Anal. Biochem. 280:103)) to amplify 3 SNPs. The SNPs were codon 72 (C/G) in the p53 gene, nucleotide position 677 (C/T) in the methylenetetrahydrofolate reductase (MTHFR) gene and nucleotide position 196 (A/G) on the glycoprotein IIIa (GP3a) gene. To allow for immobilization of the PCR products on streptavidin beads and preparation of single strand DNA, biotinylated inner PCR primers were used. The biotinylated PCR-products (~ 80 bp) were immobilised onto streptavidin-coated paramagnetic beads (Dynabeads® M-280, Dynal, Oslo, Norway) and by strand-specific elution a pure template for extension was obtained. Briefly, 100 µl of the PCR-products was captured by incubation for 15 minutes at room temperature with 5 mg/ml of beads in 100 µl binding/washing buffer (10 mM Tris-HCl (pH 7.5) 1 mM EDTA, 2 M NaCl, 1 mM ß-mercaptoethanol, 0.1 % Tween® 20). After washing and removal of supernatant, the strands were separated by incubation with 4 µl of 0.1 M NaOH for 5 minutes. The alkaline supernatant with the non-biotinylated strand was neutralised with 2.2 µl of 0.17 mM HCl and 1 µl of 100 mM Tris-Acetate pH 7.5, 20 mM $MgAc_2$.

Extension and hybridization

Single strand DNA:

The single strand DNA prepared above was divided into two aliquots and 2.5 pmoles of allele specific primers were annealed by incubation at 72 °C for 5 minutes in a volume of 20 µl. The annealed primer-DNA template was then further divided into two separate

reactions for direct comparison of extension with and without apyrase. When multiplex extension was performed, single strand DNA from the 3 templates was mixed and allele specific primers were annealed.

Extension was performed in solution on 10 μl of the annealed DNA (corresponds to 25 ul of each PCR product) in a 60 μl volume containing 100 mM Tris-Acetate, 0.5 mM EDTA and 5 mM Mg-acetate with 1.4 μM pmoles of cy5 labelled dNTPs, 2.5 μg BSA, 1.25 mM DTT, 10 Units exonuclease-deficient (exo-) Klenow DNA polymerase and optionally 8 mU apyrase. Following incubation of the extension products at room temperature for 15 minutes, 60 μl 10X SSC/0.4% SDS was added to the extension reactions and 100 :l was then hybridized to the oligonucleotide microarray. Hybridization was performed on the GeneTAC hybridization station (Genomic solutions, MI, USA) at 50 °C for 20 minutes followed by washing in 2X SSC/0.1% SDS for 5 minutes proceeded by washing in 0.6X SSC for a further 5 minutes. The slides were briefly rinsed in H_2O and dried by centrifugation at 500g for 1 minute.

Double strand DNA:

One hundred and sixty microlitres of PCR product was treated with 16 Units of calf alkaline phosphatase and 32 Units exonuclease I at room temperature for 30 minutes. The enzymes were inactivated by incubation at 95 °C for 12 minutes and the DNA was divided into two aliquots and annealed to 5 pmoles of allele specific primers (incubation at 95° C for 2 minutes followed by incubation at 72 °C for 5 minutes) in a volume of 100 μl. The annealed primer-DNA template was then further divided into two separate reactions for direct comparison of extension with and without apyrase. Extension was carried out on 50 μl of annealed double strand DNA (corresponds to 40 μl PCR product) in a 100μl as described above. Following incubation at RT for 15

minutes, 25 μl 20X SSC and 12 μl 10% SDS was added to the extension mixture and 100 μl was hybridized to the slide as described above.

Data analysis

The slides were scanned at optimal laser/PMT values using the GMS 417 scanner (Affymetrix, USA) and the features quantitated using GenePix 2.0 software (Axon Instruments, USA). Since different extension/hybridization experiments were compared, the micorarray data was subjected to a normalization procedure. This involved including a 66 mer oligonucleotide control and 18 mer extension probe (Table 3) together with the target DNA that was subjected to extension and hybridization. Since the intensity of this control should be constant from slide to slide it was used to normalize the slides for comparative purposes. Extension ratios were calculated by taking the ratio of the high versus the low signal.

Results

Results of AMASE for simultaneous genotyping of 3 SNPs on DNA microarrays are shown in Table 4. Inclusion of apyrase resulted in the correct genotype being called in all cases. However when apyrase was omitted, SNPs were incorrectly genotyped in 3 samples (illustrated in boldface type) if the criteria of a ratio ≥ 2.5 is required to call a homozygous genotype and ≤1.5 for a heterozygous sample. Two replicates of each feature were spotted which allows an estimation of the variability of the method and the standard deviation for each feature is shown in Table 4. The results in Table 4 are based on extension of single strand DNA while preliminary results for genotyping of double strand DNA gave ratios of 3.9±0.2 (with apyrase) versus 2.7±0.4 (without apyrase) for codon 72 of the p53 gene (sample g1).

Table 3. Sequence of capture and extension probes

SNP	Probe	Sequence (5'-3')
P53	Capture probe	TGA AGC TCC CAG AAT GCC
	Extension 1	AGA GGC TGC TCC CCC
	Extension 2	AGA GGC TGC TCC CCG
MTHFR	Capture probe	CAG CCT CAA AGA AAA GCT
	Extension 1	GCG TGA TGA TGA AAT CGG
	Extension 2	GCG TGA TGA TGA AAT CGA
GPIIIa	Capture probe	CTT CTC TTT GGG CTC CTG
	Extension 1	TCT TAC AGG CCC TGC CTC C
	Extension 2	TCT TAC AGG CCC TGC CTC T
Control	Capture probe	GGT GCA CGG TCT ACG AGA
	Extension probe	CCT CCC GGG GCA CTC GCA
	Extension template	AGG CCT TGT GGT ACT GCC TGG TAG GGT GCT TGC GAG TGC CCC GGG AGG TCT CGT AGA CCG TGC ACC

Table 4. Genotyping of SNPs on microarrays using AMASE

SNP	Sample	Sequencing Result	Allele-Specific Extension Ratio(high /low)	
			Microarray	
			- apyrase	+ apyrase
p53 (G/C)	g1	G/G	1.9±0.2 *	g1
	116	G/C	2.5±0.5*	116
	123	C/C	22.1±0.5	123
THFR (C/T)	1001	C/C	19.8±1.8	1001
	1055	T/C	nd	1055
	1004	T/T	21.3±3.7	1004
GPIIIa (G/A)	1267	G/G	2.2±0.2	1267
	1001	G/A	nd	1001
	1055	A/A	8.6±0.7	1055

*indicates that these samples were not genotyped in a multiplex format nd (not determined) indicates that these samples have yet to be tested

Example 3
Double Stranded DNA Analysis of a SNP

Materials and Methods

PCR amplification of wiaf 1764 was performed as described in the foregoing examples. In order to perform double-strand DNA analysis of this SNP (without strand separation by the use of beads), the excess of primers, nucleotides and the released PPi in the PCR had to be removed. For this purpose, the enzymes shrimp alkaline phosphatase (4 U) (Roche Diagnostics) and E. *coli* exonuclease I (8 U) (Amersham Pharmacia Biotech, Uppsala, Sweden) were added to 40 µl of each PCR product. Shrimp alkaline phosphatase was used to degrade PPi and the dNTPs while exonuclease I removed single-stranded DNA molecules including PCR primers. The enzymatic degradation was allowed to proceed for 30 min at room temperature. The mixtures were then heated to 97° C for 12 min to deactivate the enzymes. The samples were divided into two tubes (20 µl in each) and pairs of allele-specific primer (0.25 µM) (one complementary to the forward strand and one complementary to the reverse strand) were added into each tube. The sequence of primers was A-forward TACAACACTCCCTTCAGATCA, A-reverse TACCATTTGTTAAGCTTTTGT, C-forward TACA ACACTCCCTTCAGATCC and C-reverse ACCATTTGTTAAGCTTTTGG. The primer pairs in different tubes differed only in their 3'-ends (underlined bases indicate the alternating 3'-ends) to allow discrimination by allele-specific extension using DNA polymerase. After addition of primers, the samples were incubated at 97° C for 2 min and then cooled to room temperature, allowing hybridization of allele-specific primer pairs. The content of each well was then further divided into two separate reactions for comparison of extension analysis with and without apyrase. Extension and real-time luminometric monitoring was performed at

25°C in a Luc96 pyrosequencer instrument (Pyrosequencing, Uppsala, Sweden). An extension reaction mixture was added to the samples (10 µl) with annealed primers (the substrate) to a final volume of 50 µl. The extension reaction mixture contained 10 U exonuclease-deficient (exo-) Klenow DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden), 4 µg purified luciferase/ml (BioThema, Dalarö, Sweden), 15 mU recombinant ATP sulfurylase, 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA, 5 mM Mg-acetate, 0.1% (w/v) bovine serum albumin (BioThema), 1 mM dithiothreitol, 10 µM adenosine 5'-phosphosulfate (APS), 0.4 mg polyvinylpyrrolidone /ml (360 000), 100 µg D-luciferin/ml (BioThema) and 8 mU of apyrase (Sigma Chemical Co., St. Louis, MO, USA) when applicable. Prior to nucleotide addition and measuring of emitted light, pyrophosphate (PPi) was added to the reaction mixture (0.08 µM). The PPi served as a positive control for the reaction mixture as well as peak calibration. All the four nucleotides (Amersham Pharmacia Biotech, Uppsala, Sweden) were mixed and were dispensed to the extension mixture (0.8 µM, final concentration). The emitted light was detected in real-time.

Results and Discussion

Figure 6 shows the results of bioluminometric analysis of wiaf 1764. The SNP wiaf 1764 has the variants G and/or T (C and/or A). Two pairs of allele-specific primers were used to analyze this SNP, one complementary to the forward strand and one complementary to the reverse strand. The primer pairs differed in their 3'-ends to allow discrimination by allele-specific extension using DNA polymerase, (extension 1 and extension 2 in Figure 6). Prior to hybridization of allele-specific primers the PCR products were treated by shrimp alkaline phosphatase and exonuclease I to remove the excess of primers,

nucleotides and PPi. This allowed heat separation of double-stranded DNA and direct analysis of the PCR product without strand separation by using beads. Thus, allele-specific extension was performed on both strands of a double-stranded DNA. The bioluminimetric assay was performed without using apyrase (- apyrase in Figure 6) and by the use of apyrase (+ apyrase in Figure 6). All three variants of wiaf 1764 were analyzed and the ratios between the match and mismatch signals were calculated. The ratios are outlined in the bottom of Figure 6. As it is shown all variants of the SNP could correctly be scored when apyrase was used in the system (ratios 4, 1 and 4.1 for samples g011, 119 and g055 respectively) while two of the same samples were wrongly scored when apyrase was not applied (ratios 1.5 and 1.2 for samples g011 and g055 respectively).

The advantage of using two primers in AMASE is that these can be utilized in a PCR amplification assay when a thermostable nucleotide-degrading enzyme (e.g. alkaline phosphatase) is available. A perfectly match primer pair will give rise to amplification while mismatch primer pair will not. After amplification, the PCR products are directly analyzed by a luminometric assay since the same primer pair is used in allele-specific extension. Another advantage of using two allele-specific primers is that the sensitivity increases by a factor of two because signals of two extensions are obtained instead of one.

Example 4

Introduced mismatch in allele-specific primers to improve allele-specific extension

The present example describes a method of allele-specific extension using apyrase and an introduced mismatch in the allele-specific primer, with non-stringent conditions for extension.

PCR amplification of GPIIIa gene was performed as described in the foregoing examples. The SNP in the GPIIIa gene has the variants C/T (G/A). The PCR products were immobilized on magnetic beads and single-stranded DNA was obtained by alkaline treatment as described in the foregoing examples. The immobilized single-stranded DNA was used as target template in the assay. Two different primers were designed. The sequence of primers was CTG TCT TAC AGG CCC TGC CTG CG for GPIIIC and CTG TCT TAC AGG CCC TGC CTG TG for GPIIIT. The immobilized strand was resuspended in 32 μl H_2O and 4 μl annealing buffer (100 mM Tris-acetate pH 7.75, 20 mM Mg-acetate). The single stranded DNA was divided into two parallel reactions in a microtiter-plate (18 μl/well) and 0.2 μM (final concentration) of primers were added to the single stranded templates in a total volume of 20 μl. The primers were designed so that the 3'-end was one base after the SNP site and was complementary to the target DNA (indicated in *italics*). Thus, the base before the 3'-end (3'-1) was complementary to the SNP variants and was the only difference between the two allele-specific primers (underlined bases). The base before the SNP site (3'-2) on both primers (indicated in **bold**) was an introduced mismatch to the target DNA (**G** on the primers and **G** on the target DNA). Therefore, when the allele-specific base in the primer does not match to the target template, two mismatches (positions 3'-1 and 3'-2) and one match (position 3') will be made between the template and the last 3 bases in the primer (Fig. 7). When the allele-specific base in the primer does match to the target template, the last two bases in the 3'-end of the primer (positions 3' and 3'-1) will be complementary to the target DNA while the introduced mismatch (position 3'-2) is not complementary. Raw-data were obtained from a luminometric assay. The conditions (enzymes, substrates etc) of performance of the

luminometric assay are as described in the foregoing examples. Prior to nucleotide addition and measurement of emitted light, pyrophosphate (PPi) was added to the reaction mixture (0.02 μM). The PPi served as a positive control for the reaction mixture as well as peak calibration. All three variants of the SNP were investigated with (+ apyrase) and without (- apyrase) addition of apyrase (Fig. 7). As shown in Fig. 7, Sample 1055 is homozygous A in the target DNA template. Thus, an allele-specific primer containing the base C at 3'-1 position is a mismatch to the target (C to A mismatch) and since the 3'-2 position is also a mismatch (G to G mismatch), the two mismatches disrupt the hydrogen bind of the match base at the 3' position of the primer (G to C match) and no extension is observed. When the allele-specific primer contains a matching base to the target DNA at the 3'-1 position (T to A match), two complementary bases are made (at 3'-1 and 3' position) between the primer and template. In a non-stringent extension condition (extension at 25° C), the mismatch base at position 3'-2 does not remove the two complementary bases at 3'-1 and 3', which leads to extension. The same explanation can be used for sample 1267 that is homozygous G. In the heterozygous case (sample 1001) both allele-specific bases are complementary to the respective variant and give rise to extension signals.

Example 5

Apyrase Mediated Allele Specific Extension on Microarray for Mutation Detection

In this example, a microarray assay was used to detect unknown mutations in a 15 base stretch in exon 5 of the p53 tumour suppressor gene. Partially overlapping extension primers were designed, but instead of two variants differing only in the 3'-end nucleotides

as for SNPs, all four possible variants of 3'-end were used (Figure 8). This allowed characterization of all possible mutations in a given position instead of two allelic variants. Since four partially overlapping primers are needed for detection and characterization of mutations in a position, 60 oligonucleotides were spotted on the chip for analysis of 15 bases. These extension primers were then allowed to hybridize to template, followed by extension *in situ* using fluorescent nucleotides. After the extension the microarray was scanned and fluorescent intensities were measured and compared. Four different templates were used: a 100% WT template and three mutated templates in a 50% mixture with WT template.

Materials and Method

Oligonucleotides

The allele specific extension primers were synthesized with a 5' amino group, which facilitates covalent immobilization on the glass slide. A spacer sequence of 15 T residues was included at the 5' end of the gene specific sequence that contained 18 nucleotides (Table 5). The gene specific sequences of the extension primers were designed to have a Tm of 62-64^{0}C with the 3'-nucleotide hybridizing at the variant position. In addition, amino linked oligonucleotides, which are printed on the arrays to act as hybridization and extension controls, were synthesized (Table 6). A labeled hybridizing oligonucleotide, which was used for normalization, was synthesized with a Cy3 label (Table 6). A 66-mer control extension target, which hybridizes to the spotted extension control, was also synthesized (nucleotides 274-341 of the HCV genome, accession number M623321) (Table 6). Synthetic samples of 60-mers (nucleotides 13205-19 of the p53 gene, accession number U94788) were used to hybridize to the allele specific extension primers (Table 7). These were either of wild-

type sequence or containing one mutation. The oligonucleotides were synthesized by Thermo Hybaid, Interactiva, (Germany) and MWG-Biotech, (Germany).

Table 5. Gene specific extension primers.

3'-Position	Sequence	3'-Position	Sequence
205T	$(T)_{15}$CGGAGGTTGTGAGGCGCT	212G	$(T)_{15}$TGTGAGGCGCTGCCCCCG
205A	$(T)_{15}$CGGAGGTTGTGAGGCGCA	212T	$(T)_{15}$TGTGAGGCGCTGCCCCCT
205C	$(T)_{15}$CGGAGGTTGTGAGGCGCC	213C	$(T)_{15}$GTGAGGCGCTGCCCCCAC
205G	$(T)_{15}$CGGAGGTTGTGAGGCGCG	213A	$(T)_{15}$GTGAGGCGCTGCCCCCAA
206G	$(T)_{15}$GGAGGTTGTGAGGCGCTG	213G	$(T)_{15}$GTGAGGCGCTGCCCCCAG
206A	$(T)_{15}$GGAGGTTGTGAGGCGCTA	213T	$(T)_{15}$GTGAGGCGCTGCCCCCAT
206C	$(T)_{15}$GGAGGTTGTGAGGCGCTC	214C	$(T)_{15}$TGAGGCGCTGCCCCCACC
206T	$(T)_{15}$GGAGGTTGTGAGGCGCTT	214A	$(T)_{15}$TGAGGCGCTGCCCCCACA
207C	$(T)_{15}$GAGGTTGTGAGGCGCTGC	214G	$(T)_{15}$TGAGGCGCTGCCCCCACG
207A	$(T)_{15}$GAGGTTGTGAGGCGCTGA	214T	$(T)_{15}$TGAGGCGCTGCCCCCACT
207G	$(T)_{15}$GAGGTTGTGAGGCGCTGG	215A	$(T)_{15}$GAGGCGCTGCCCCCACCA
207T	$(T)_{15}$GAGGTTGTGAGGCGCTGT	215C	$(T)_{15}$GAGGCGCTGCCCCCACCC
208C	$(T)_{15}$AGGTTGTGAGGCGCTGCC	215G	$(T)_{15}$GAGGCGCTGCCCCCACCG
208A	$(T)_{15}$AGGTTGTGAGGCGCTGCA	215T	$(T)_{15}$GAGGCGCTGCCCCCACCT
208G	$(T)_{15}$AGGTTGTGAGGCGCTGCG	216T	$(T)I_5$AGGCGCTGCCCCCACCAT
208T	$(T)_{15}$AGGTTGTGAGGCGCTGCT	216A	$(T)_{15}$AGGCGCTGCCCCCACCAA
209C	$(T)_{15}$GGTTGTGAGGCGCTGCCC	216C	$(T)_{15}$AGGCGCTGCCCCCACCAC
209A	$(T)_{15}$GGTTGTGAGGCGCTGCCA	216G	$(T)_{15}$AGGCGCTGCCCCCACCAG
209G	$(T)_{15}$GGTTGTGAGGCGCTGCCG	217G	$(T)_{15}$GGCGCTGCCCCCACCATG
209T	$(T)_{15}$GGTTGTGAGGCGCTGCCT	217A	$(T)_{15}$GGCGCTGCCCCCACCATA
210C	$(T)_{15}$GTTGTGAGGCGCTGCCCC	217C	$(T)_{15}$GGCGCTGCCCCCACCATC
210A	$(T)_{15}$GTTGTGAGGCGCTGCCCC	217T	$(T)_{15}$GGCGCTGCCCCCACCATT
210G	$(T)_{15}$GTTGTGAGGCGCTGCCCG	218A	$(T)_{15}$GCGCTGCCCCCACCATGA
210T	$(T)_{15}$GTTGTGAGGCGCTGCCCT	218G	$(T)_{15}$GCGCTGCCCCCACCATGC
211C	$(T)_{15}$TTGTGAGGCGCTGCCCCC	218C	$(T)_{15}$GCGCTGCCCCCACCATGG
211A	$(T)_{15}$TTGTGAGGCGCTGCCCCA	218T	$(T)_{15}$GCGCTGCCCCCACCATGT
211G	$(T)_{15}$TTGTGAGGCGCTGCCCCG	219G	$(T)_{15}$CGCTGCCCCCACCATGAG
211T	$(T)_{15}$TTGTGAGGCGCTGCCCCT	219A	$(T)_{15}$CGCTGCCCCCACCATGAA
212A	$(T)_{15}$TGTGAGGCGCTGCCCCCA	219C	$(T)_{15}$CGCTGCCCCCACCATGAC
212C	$(T)_{15}$TGTGAGGCGCTGCCCCCC	219T	$(T)_{15}$CGCTGCCCCCACCATGAT

Table 6. Control oligonucleotides.

Sequence	Use
5'-CCTGGCAAATTCATTTCT-3'	Printed hybridization control
5'-GGTGCACGGTCTACGAGA-3'	Hybridized hybridization control
5'-AGAAATGAATTTGCCAG G-3'	Printed extension control
See text	Hybridized extension control

Table 7. Hybridized oligonucleotides.

Sequence	Mutation
ACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGCAGCGCCTCACAA CCTCCGTCATGTG	WT
ACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGAAGCGCCTCACAA CCTCCGTCATGTG	206 G to T
ACCATCGCTATCTGAGCAGCGCTCATGGTG~GGCAGCGCCTCACAA CCTCCGTCATGTG	210 C to A
ACCATCGCTATCTGAGCAGCGCTCATGGAGGGGGCAGCGCCTCACAA CCTCCGTCATGTG	212 A to T

Microarry preparation

Amino linked oligonucleotide capture probes suspended at a concentration of 20 μM in 150 mM sodium phosphate pH 8.5 and 0.06% sarkosyl were spotted using a GMS 417 arrayer (Affymetrix, USA) or a Genetix arrayer equipped with solid pins (Genetix, UK) on 3D-link activated slides (Motorola Life Sciences, IL, USA). Sarkosyl was added to the spotting solution as it improved spot uniformity. The arrays were printed in 2-3 different areas on the slides with either 1 or 4

hits/dot and with 2-4 replicate spots in each array. Prior to spotting the extension primer solutions were spiked with 2 μM of the 18-mer amino linked hybridization control oligonucleotide, which hybridizes to a labeled oligonucleotide with the duplex serving as a hybridization and normalization control. The second 18-mer oligonucleotide, which acts as an extension control, was also printed on the slide. This oligonucleotide hybridizes to a 66-mer extension control target oligonucleotide (mixed with the sample DNA) and the duplex serves as a positive control for extension. After printing, the arrays were incubated overnight in a humid chamber followed by post coupling as outlined by the manufacturer. Briefly the slides were incubated at 50°C for 15 minutes in blocking solution (50 mM ethanol amine, 0.1M Tris pH 9 and 0.1% SDS), rinsed twice in dH_2O and washed with 4X SSC/0.1% SDS (prewarmed to 50°C) for 15 to 60 minutes. The arrays were then rinsed in H_20 and dried by centrifugation for 3 minutes at 800rpm.

Allele specific extension

4 pmol of synthetic single stranded 60-mers, either 100% wild-type or mixed equally to mutated oligonucleotides (see Table 6), was incubated in 150 μl 5XSSC/0.2% SDS with 0.5 pmoles synthetic DNA (66 mer extension control), 0.12 pmoles Cy3 labeled hybridization control and 0.5 μg SSB (Amersham Pharmacia, Biotech, Uppsala, Sweden) at 70°C for 5 minutes. 140 μl of this solution was then hybridized to the microarray in a GeneTAC hybridization station (Genomic Solutions, MI, USA). Hybridization was performed at 58°C for 20 minutes followed by two washes in 2X SSC/0.1% SDS (preheated to 58°C) for 5 minutes proceeded by washing in 0.2X SSC and 0.1X SSC for 1 minute. The slides were dried by centrifugation at 800 rpm for 1 minute prior to scanning with the Cy5 laser.

To perform extension, 100 μl of an extension mix

was prepared containing 80 mM Tris-Acetate, 0.4 mM EDTA, 1.4 mM $MgAc_2$, 0.5 mM DTT, 2 μg *E. coli* tRNA, 1 μg SSB, 20 units exonuclease-deficient (exo-) Klenow DNA polymerase and 8 mU apyrase. Immediately prior to extension, 40 μl of 2.5 μM dATP, dGTP, dTTP and Cy5 labeled dCTP (Amersham Pharmacia BioTech) was added to the extension mix and the solution injected over the slide. Extension was performed for 15 minutes at 28°C followed by a washing procedure performed as described above.

Scanning and data analysis after extension

The slides were scanned using the GMS 418 scanner (Affymetrix, SA). Generally the optimal parameters were laser and photomultiplier tube (PMT) settings of 100 % and 50% respectively for Cy5 and laser and PMT settings of 100% and 50% for Cy3. Sixteen-bit TIFF images were then imported into GenePix 2.0 software (Axon Instruments, USA) for analysis. After the median local background was subtracted from the mean signal of the spots, means for the 8 spots of each allele specific extension primer printed on each slide were calculated. The Cy3 channel signal was used as a visual aid as to see if the hybridization were evenly distributed on the chip.

Results and Discussions

This example demonstrates the use of the micro array assay to detect unknown mutations in a 15-base stretch in exon 5 of the p53 tumour suppresser gene. Partially overlapping extension primers were designed, but instead of two variants differing only in the 3'-end nucleotides as for SNPs, all four possible variants of 3'-end were used (Figure 8). This was to allow for all three possible mutations instead of two allelic variants. To perform detection and characterization of mutations in these 15 bases, 60 partially overlapping oligonucleotides were spotted on the chip. These

extension primers were then allowed to hybridise to a synthetic 60-mer oligonucleotide template, followed by extension in *situ* using fluorescent nucleotides. After the extension the microarray was scanned and fluorescent intensities were measured and compared (Figure 9). Four different templates were used - 100% WT template and three mutant templates in a 50% mixture with the WT template (Table 7).

Averages of the intensities of the 8 replicates on each slide are shown as diagrams in Figure 9. These intensities were used to calculate the ratios between the match signal and the mismatch signal.

This example demonstrates that unknown mutations can be detected by apyrase mediated allele specific extension.

Claims

1. A method of detecting a base at a pre-determined position in a nucleic acid molecule, said method comprising:

performing primer extension reactions using base-specific detection primers, each primer being specific for a particular base at said predetermined position, and comparing said primer extensions to determine which base is present at said position, wherein said primer extension reactions are performed using labelled nucleotides and wherein a nucleotide-degrading enzyme is present during the primer extension reaction.

2. A method as claimed in claim 1, wherein one of said detection primers comprises a base which is complementary to the base it is desired to detect (the target base) and another detection primer is non-complementary to said target base.

3. A method as claimed in claim 2, wherein said base in the primer corresponding to the target base is at the 3' end of the primer.

4. A method as claimed in any one of claims 1 to 3 wherein the detection primers are allele-specific.

5. A method as claimed in any one of claims 1 to 4 wherein two detection primers are used.

6. A method as claimed in claim 5, wherein the first of said primers is specific for a non-mutated base at the predetermined position, and the second primer is specific for a mutated base at the predetermined position.

7. A method as claimed in claim 5, wherein the first of said primers is specific for one allele, and the second primers is specific for a different allele.

8. A method as claimed in any one of claims 1 to 4, for the detection of unknown base changes wherein four primers are used, each specific for one of the bases A, T, C or G.

9. A method as claimed in claim 8, wherein each said base-specific detection primer comprises a different base (A, T, C or G) at its 3' terminus, or at any other "base-specific" position.

10. A method as claimed in either one of claims 8 or 9, wherein a set of primers are used, each specific for a particular base at different pre-determined positions.

11. A method as claimed in any one of claims 1 to 10, wherein the primer extension reactions are compared by determining and comparing the rates of extension, or extension efficiencies, of the respective primer extension reactions.

12. A method as claimed in any one of claims 1 to 11 wherein the labelled nucleotide carries a fluorescent label.

13. A method as claimed in any one of claims 1 to 12, wherein the label is attached to the nucleotide via a cleavable linker.

14. A method as claimed in any one of claim 1 to 13 wherein the nucleotide degrading enzyme is apyrase.

15. A method as claimed in any one of claims 1 to 14 wherein the target nucleic acid is amplified before

hybridization.

16. A method as claimed in any one of claims 1 to 15, wherein the target nucleic acid is immobilized.

17. A method as claimed in any one of claims 1 to 14 wherein said method is performed in a solid phase microarray format.

18. A method as claimed in claim 17 wherein said primers are immobilised.

19. A method as claimed in any one of claims 1 to 18 wherein said primers are tagged on the 5'-ends by barcodes.

20. A method as claimed in any one of claims 1 to 19 wherein the target nucleic acid is double stranded.

21. A method as claimed in any one of claims 1, 2 or 4 to 20, wherein the said base in the primer corresponding to the target base is at the 3'-1 position in the primer (i.e. the penultimate base of the primer).

22. A method as claimed in claim 21, wherein the base at the 3' position in said primer is complementary to the base that is 5' to the predetermined position in the target, and the base in the 3'-2 (ante-penultimate) position in the primer is non-complementary to the base that is 3' to the predetermined position in the target.

23. A method for detecting an allele-specific base at a predetermined position in a target nucleic acid comprising conducting a first and a second allele-specific primer extension reaction using a first and a second primer, respectively that hybridizes to a region of said target nucleic acid, each of said primers

having: 1) a 3'-end base that is complementary to the base that is 5' of the predetermined position in the target; 2) a base one position from the 3'-end that in the first primer is complementary to a non-mutated based at the predetermined position, and in the second primer is complementary to a mutated base at the predetermined position; and 3) a base two positions from the 3'-end that is non-complementary to the base that is 3' of the predetermined position in the target; and determining primer extension efficiency in said first and second reactions, whereby greater efficiency in said first reaction is indicative of the presence of a non-mutated base at said predetermined position, and whereby greater efficiency in said second reaction is indicative of the presence of a mutated base at said predetermined position.

24. The method of claim 23 wherein said extension reactions are performed in the presence of a nucleotide degrading enzyme.

25. The method of claim 24 wherein said nucleotide degrading enzyme is apyrase.

26. The method of any one of claims 23 to 25 wherein said target nucleic acid is amplified before hybridization.

27. The method of any one of claims 23 to 26 wherein said primer extension efficiency is measured by an assay selected from mass spectroscopy, a luminometric assay, a fluorescent assay, and pyrosequencing.

28. The method of any one of claims 23 to 27 wherein said method is performed in a solid phase microarray format.

29. The method of any one of claims 23 to 28 wherein said primers are tagged at the 5'-ends by barcodes.

30. The method of any one of claims 23 to 29 wherein said target nucleic acid is double stranded.

FIG 1A

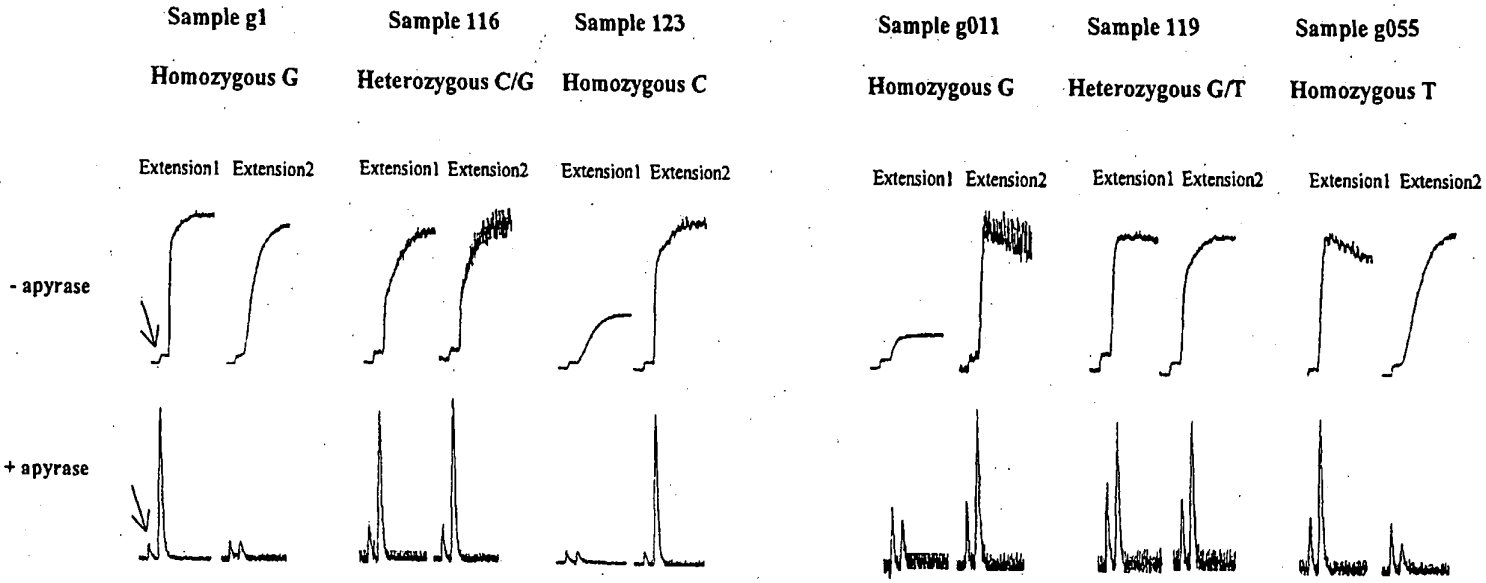
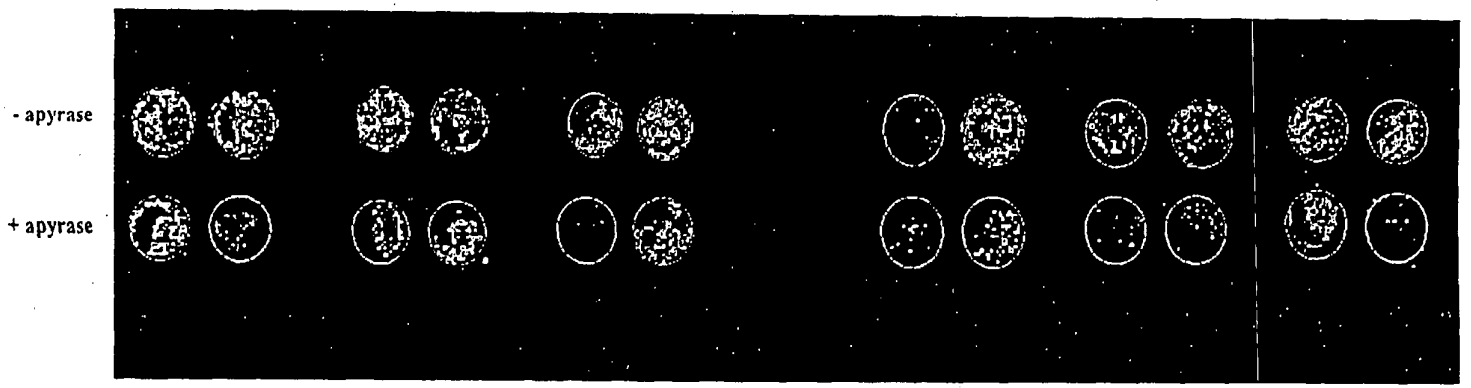

p53 codon 72
wiaf 1764
Sample g1
Homozygous G
Sample 116
Heterozygous C/G
Sample 123
Homozygous C
Sample g011
Homozygous G
Sample 119
Heterozygous G/T
Sample g055
Homozygous T
Extension1 Extension2
- apyrase
+ apyrase

FIG 1B


- apyrase
+ apyrase

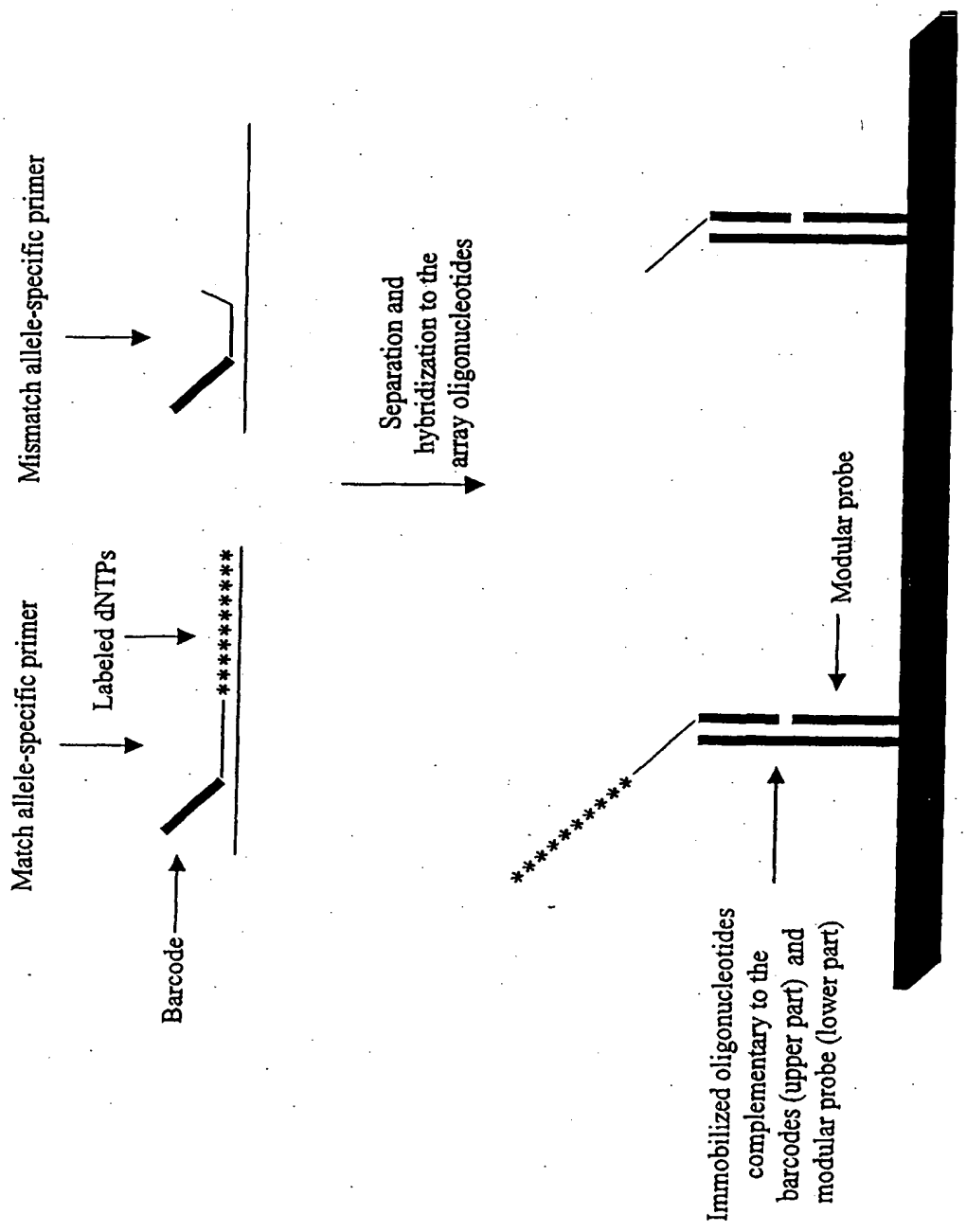

Match allele-specific primer
Labeled dNTPs
Barcode
Mismatch allele-specific primer
Separation and hybridization to the array oligonucleotides
Modular probe
Immobilized oligonucleotides complementary to the barcodes (upper part) and modular probe (lower part)

Fig 2

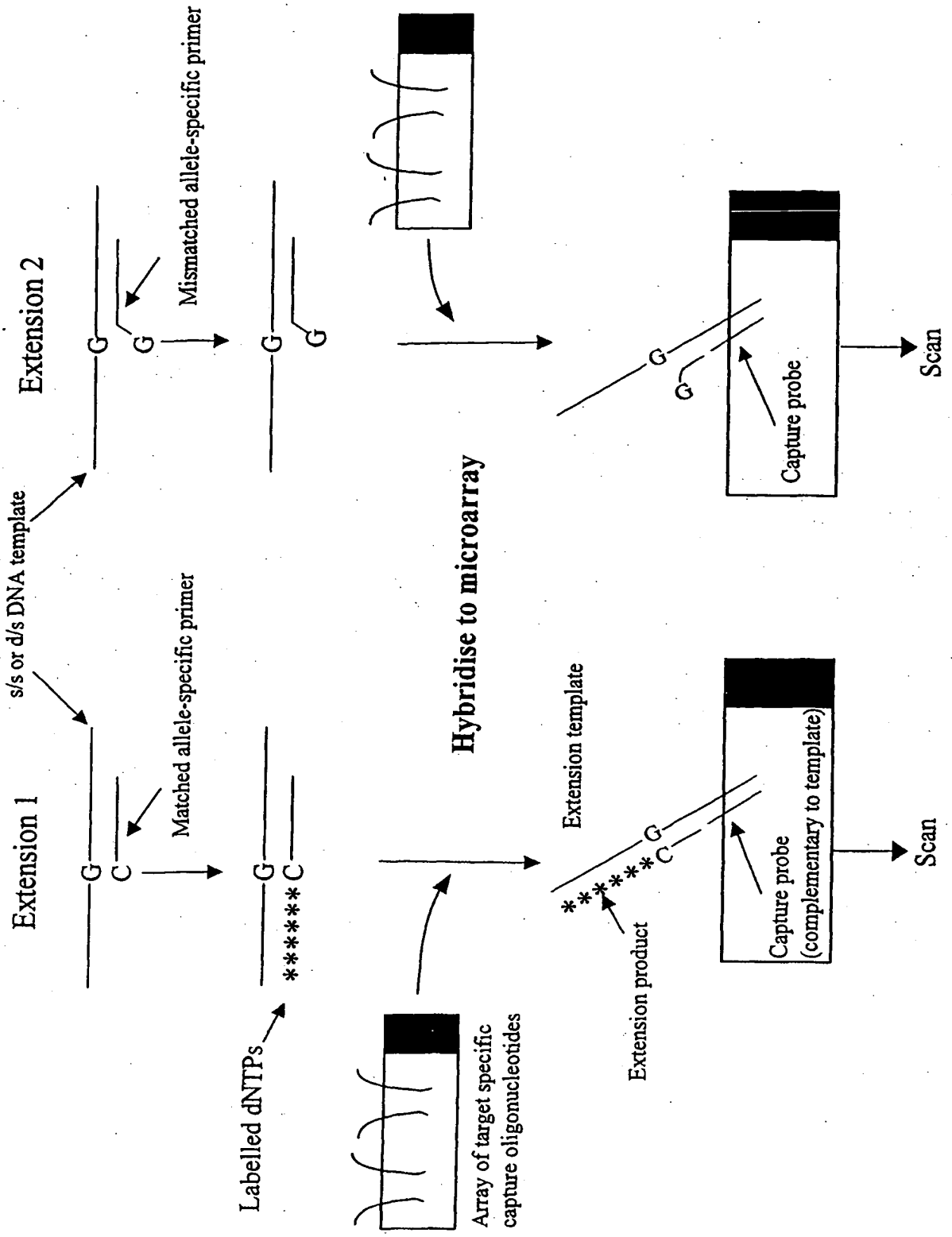

Extension 1
s/s or d/s DNA template
Matched allele-specific primer
Extension 2
Mismatched allele-specific primer
Labelled dNTPs
Array of target specific capture oligonucleotides
Hybridise to microarray
Extension template
Extension product
Capture probe (complementary to template)
Capture probe
Scan
Scan

Fig 3

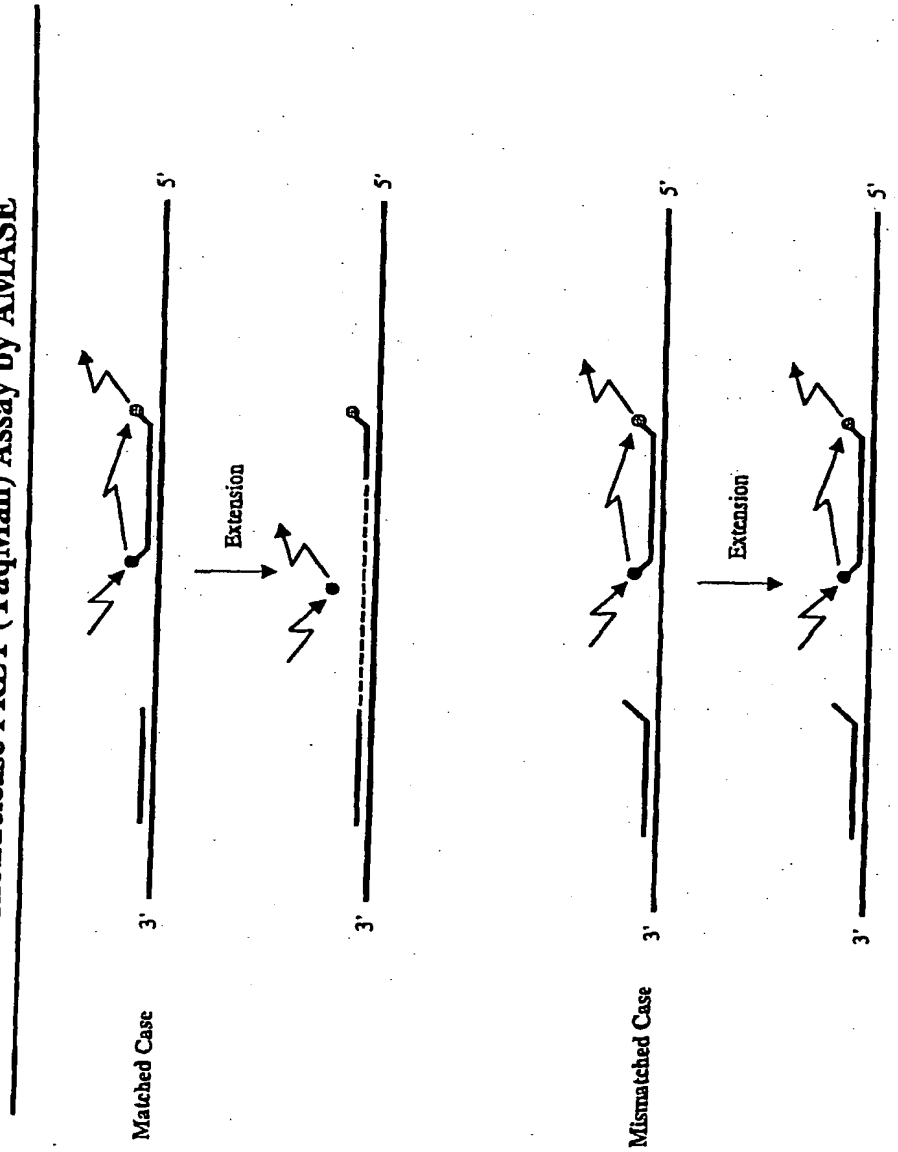
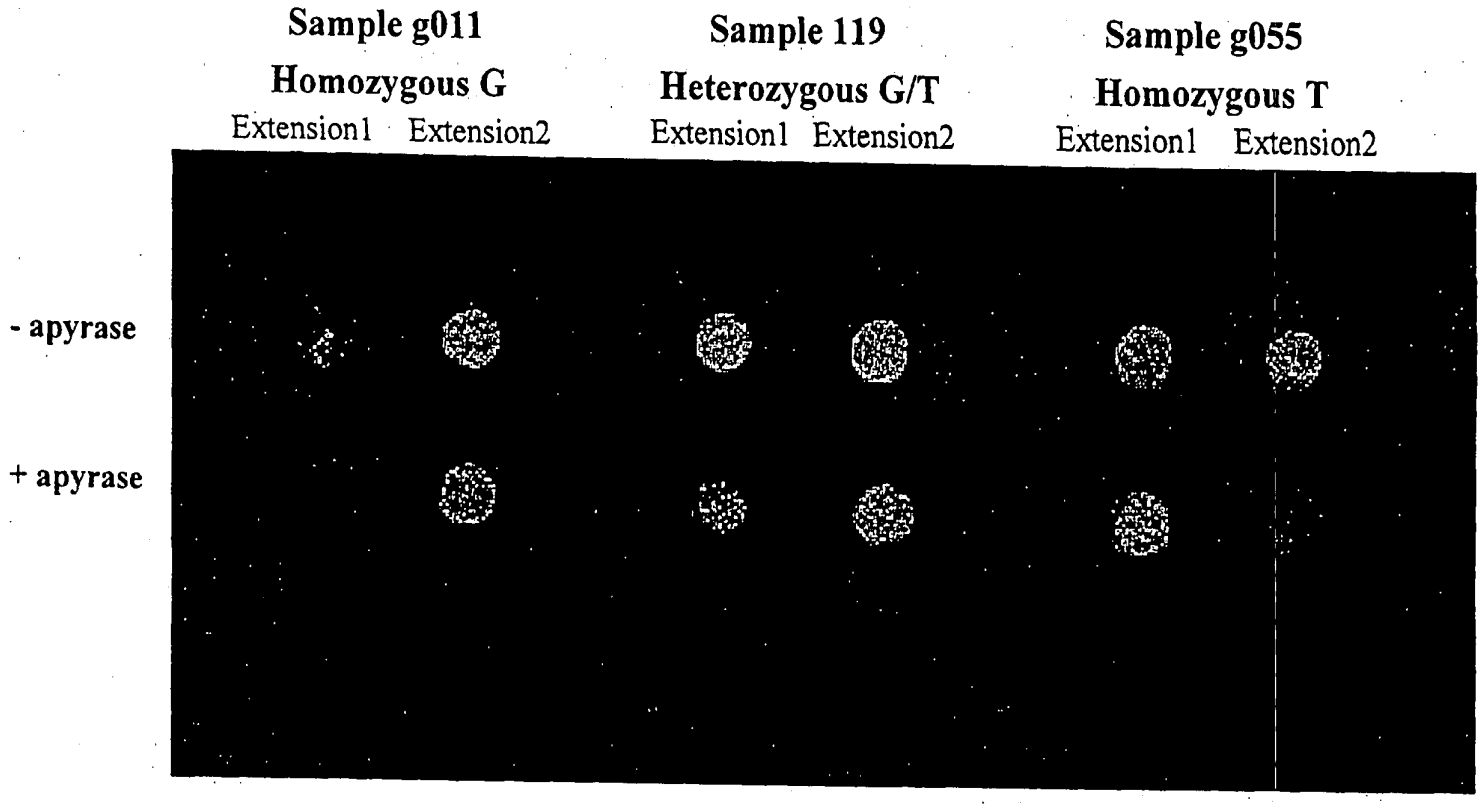

Exonuclease FRET (TaqMan) Assay by AMASE
Matched Case
Extension
Mismatched Case
Extension
5'
3'

Fig 4

wiaf 1764

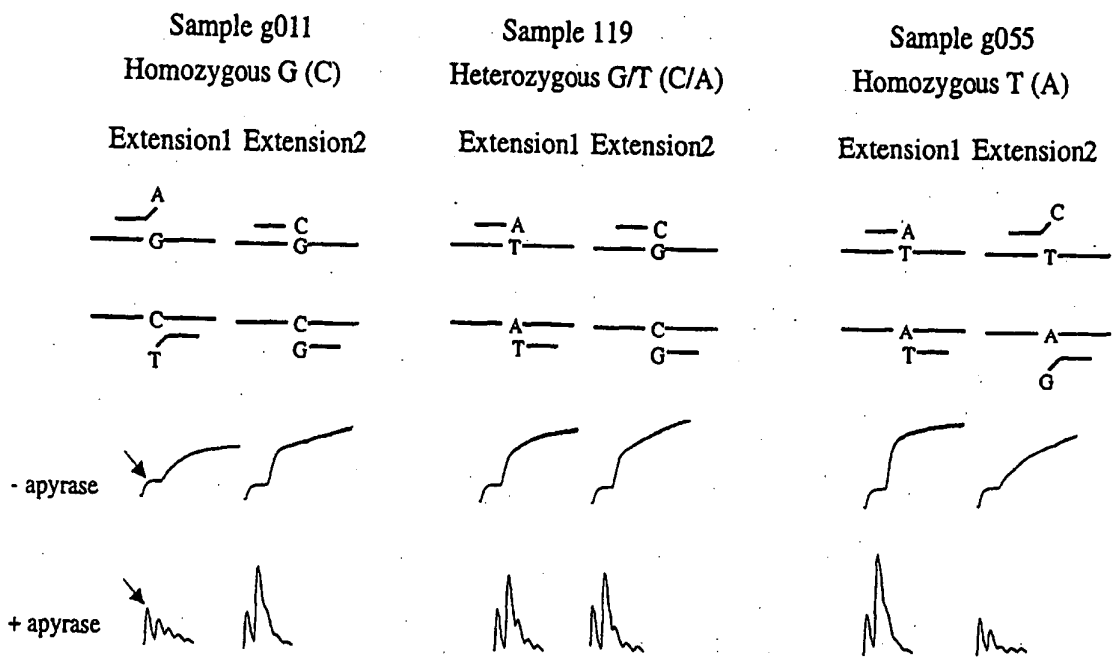


Allele-Specific Extension Ratios

- apyrase	2.5	1.2	1.6
+ apyrase	5.4	1.3	3.0

Fig 5

Bioluminometric analysis of wiaf 1764 by double allele-specific primer extension on double stranded DNA



- apyrase	1.5	1.2	1.2
+ apyrase	4	1	4.1

Fig 6

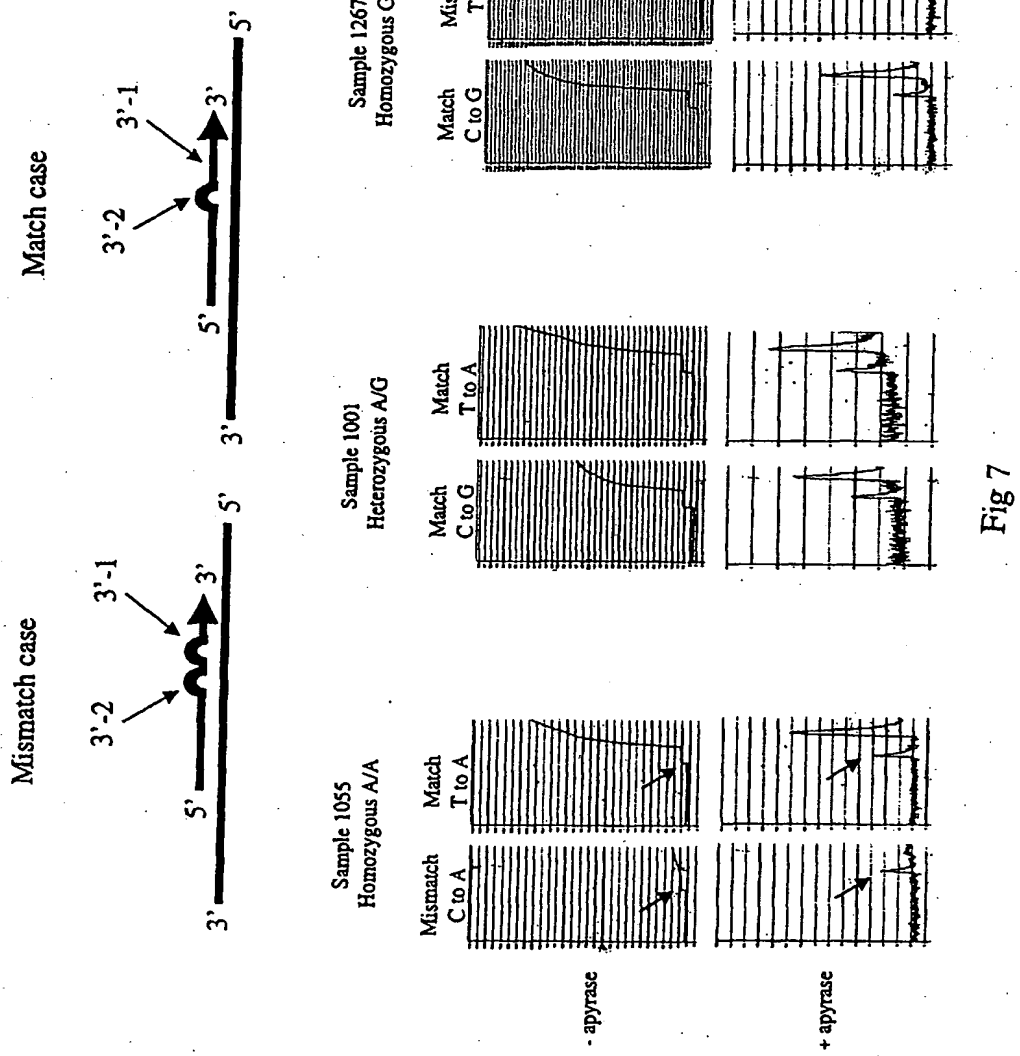

Mismatch case
Match case
3'-2
3'-1
5'
3'
Sample 1055
Homozygous A/A
Mismatch
C to A
Match
T to A
Sample 1001
Heterozygous A/G
Match
C to G
Match
T to A
Sample 1267
Homozygous G/G
Match
C to G
Mismatch
T to G
- apyrase
+ apyrase

Fig 7

Fig 8

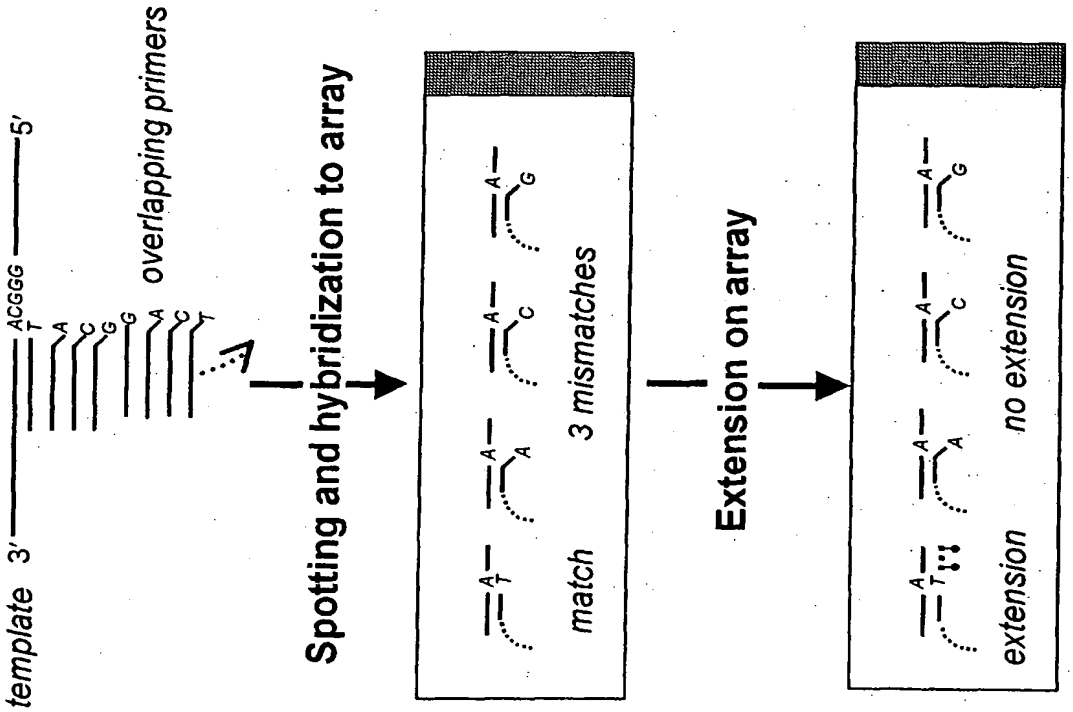

template 3'
5'
overlapping primers
Spotting and hybridization to array
match
3 mismatches
Extension on array
extension
no extension

Fig 9